DETERMINANTS OF EXERCISE-INDUCED MUSCLE DAMAGE AND THE REPEATED BOUT EFFECT IN MEN AND WOMEN

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DETERMINANTS OF EXERCISE-INDUCED MUSCLE DAMAGE AND THE REPEAT BOUT EFFECT IN MEN AND WOMEN

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ABSTRACT

Exercise induced muscle damage (EIMD) is a consequence of lengthening muscle actions, with the amount of strain placed on the muscle a key determinant of the magnitude of damage experienced. When exposed to lengthening muscle actions, skeletal muscle rapidly adapts so that when the same exercise is repeated, these markers of EIMD are attenuated, with this adaptation termed the repeated bout effect (RBE). Changes in muscle lengthening and increases in tendon compliance between exercise sessions have been hypothesised to be a mechanism underpinning the RBE, but this currently remains unclear. Moreover, muscle and tendon behaviour between two bouts of muscle damaging exercise has yet to be investigated in a female population. Therefore, the aim of this was to use US to further understand the properties and behaviour of the patella tendon (PT) and *vastus lateralis* (VL) in males and females and how these properties contribute to the EIMD response to ECC exercise and the magnitude of the repeated bout effect. In chapter 3, measures of PT cross sectional area (CSA) using ultrasonography were validated against magnetic resonance imaging. Additionally, the test-retest reliability of these measures were tested between two independent US operators. Measures of PT CSA were found to be valid and reliable and could be used interchangeably. Moreover, inter- and intra rater reliability was deemed excellent. In chapter 4, procedures for using ultrasonography to assess VL and PT behaviour at rest and during maximal exercise was shown to have excellent test-retest reliability, which did not differ between males and females. Finally, in chapter 5, it was found that VL muscle fascicle lengthening was approximately 7.6% lower following a repeated bout of lengthening exercise, and this was coupled with an attenuated EIMD response following the second bout. Additionally, this response did not differ between males, females using an oral contraceptive pill and eumenorrheic females. This work extends the understanding of muscle and tendon behaviour during repeated bouts of damaging lengthening contractions and that this behaviour is similar between males and females.

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LIST OF SYMBOLS AND ABBREVIATIONS

1RM	1 repetition maximum
ANOVA	Analysis of variance
AT	Achille's tendon
ATP	Adenosine triphosphate
BF	Biceps femoris
CK	Creatine kinase
cMVC	Maximal concentric voluntary contraction
CON	Concentric
CSA	Cross-sectional area
CV	Coefficient of variation
Cyr61	Cysteine-rich angiogenic inducer 61
DHPR	Dihydropyridine receptors
DOMS	Delayed onset of muscle soreness
DXA	Dual-energy x-ray absorptiometry scan
E-C	Excitation-contraction
ECC	Eccentric
ECM	Extra-cellular matrix
EIMD	Exercise induced muscle damage
EMG	Electromyography
FL	Fascicle length
GM	Gastrocnemius medialis
ICC	Intraclass correlation coefficient
Ig	Immunoglobulin

iMVC	Maximal isometric voluntary contraction
IOP	Index of protection
ISO	Isometric
LFF	Low-frequency fatigue
LH	Luteinising hormone
LY	Length-tension
MHC-1	Major histocompatibility complex class 1
MRI	Magnetic resonance imaging
MTC	Mid-thigh circumference
MTJ	Myotendinous junction
MTU	Muscle-tendon unit
MVC	Maximal voluntary contraction
OA	Optimal angle of force production
OCP	Oral contraceptive pill
OR	Oestrogen receptors
PA	Pennation angle
PEVK	Proline (p), lutamate (e), valine (v) and lysine (k)
PPO	Peak power output
PT	Patella tendon
PTMA	Patella tendon moment arm
RBE	Repeated bout effect
RFr	Radio frequency
RF	Rectus femoris
RFE	Residual force enhancement

RMS	Root-mean-square
RONS	Reactive oxygen and nitrogen species
SEE	Standard error of the estimate
SEM	Standard error of measurement
SICI	Short-interval intracortical inhibition
SM	Semimembranosus
SR	Sarcoplasmic reticulum
ST	Semitendinosus
TE	Typical error
TMS	Transcranial magnetic stimulation
TNC	Tenascin c
TSP-1	Thrombospondin-1
US	2D B-mode ultrasonography
VA	Voluntary activation
VAS	Visual analogue scale
VL	Vastus lateralis
YM	Young's modulus

DECLARATION

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas, and contributions from the work of others.

Any ethical clearance for the research in this thesis has been approved. Approval has been sought and granted by the Faculty of Health and Life Sciences Ethics committee for each study.

Name: Steven Marshall

Signature:

Date: 12th August 2020

CHAPTER 1 INTRODUCTION

1-1 INTRODUCTION

Exercise induced muscle damage (EIMD) of varying magnitudes will be experienced by most people at some point in their life, and likely on several occasions. EIMD is generally a consequence of movements the individual is unaccustomed to (Clarkson and Hubal, 2002) and produces a number of symptoms, most commonly, feelings of muscle soreness, although this does not always reflect the amount of damage that has occurred due to a lack of correlation between muscle soreness and other markers of EIMD (Nosaka et al., 2002). The types of movements most likely to induce EIMD involve muscle actions that require a large eccentric (ECC) component, which occur when the momentary force produced by the muscle is itself less than the force being applied to the muscle, which results in the forced lengthening of the muscle-tendon unit (MTU) (Lindstedt et al., 2002). In comparison to other types of contractions, ECC contractions consistently result in the most severe symptoms of EIMD (Hody et al., 2019, Newham et al., 1986). These symptoms can be quantified and measured to give an indication of the extent of EIMD experienced. These measures are known as markers of EIMD, the most invasive of which requires direct sampling of muscle tissue using biopsy techniques. Indirect measurements of EIMD are more commonly employed, such as the measurement of the impairment of muscle function, muscle soreness, swelling, and the leakage of intramuscular proteins into the blood (Warren et al., 1999), though this is not an exhaustive list. Several factors might dictate the severity of the EIMD experienced by a person. As highlighted earlier, contraction type is one factor but other determinants of EIMD magnitude include the speed and force of the contraction, the volume of exercise performed and which limb is exercised (Hyldahl et al., 2017, Chen et al., 2019).

An additional determinant of EIMD is the length of which muscle contracts eccentrically, where exercise at longer muscle lengths typically results in higher levels of damage than exercise at shorter muscle lengths (Nosaka et al., 2005a). Eccentric exercise at longer muscle lengths expose the muscle fibres to a high level of strain, which can result in EIMD (Lieber and Friden, 1993, Hoffman et al., 2014). Levels of strain can be measured in human skeletal muscle by using real-time 2D B-mode ultrasonography (US) to track the length of muscle fascicles at rest and during movement (Fukunaga et al., 1997, Guilhem et al., 2011, Reeves and Narici, 2003). Indeed, using this technique, the amount that a muscle fascicle lengthens during ECC exercise has been shown to correlate with markers of EIMD in the plantar flexors (Guilhem et al., 2016) and knee extensors (Hicks et al., 2017). However, the muscle fascicle is part of the MTU and therefore the role of the tendon must be considered when assessing muscle behaviour. The behaviour of both the tendon and the muscle fascicle can be measured independently during contractions (Fukunaga et al., 1997, Reeves and Narici, 2003) and research has shown that the behaviour of the tendon can indeed mediate the behaviour of the muscle fascicle, as more compliant tendons have led to reduced muscle fascicle lengthening in the plantar flexors (Guilhem et al., 2016) and knee extensors (Hicks et al., 2013). The increased use of US is uncovering more evidence to suggest that MTU behaviour is intrinsically linked to the magnitude of EIMD following ECC exercise. As with any measurement tool, the reliability and validity of US is dependent on a number of factors, notably, scanning technique, user experience and the standardisation of the scanning protocol (Van Hooren et al., 2020, McAuliffe et al., 2017). Therefore, when assessing MTU behaviour using US, the

accuracy of the measurement tool must be established, particularly when repeated measures are used.

A remarkable feature of skeletal muscle is its ability to regenerate and adapt to muscle damaging exercise, so much so that when the same exercise is repeated in the days and weeks after the first exercise, muscles generally do not succumb to the same magnitude of damage. This adaptation is known as the repeated bout effect (RBE) (Nosaka and Clarkson, 1995) and is a key theme in this thesis. Several theories have attempted to explain this adaptation that occurs between two bouts of damaging exercise, including neuromuscular adaptations, a modified inflammatory response, extracellular matrix (ECM) remodelling and changes in the behaviour of the MTU (McHugh et al., 1999a, McHugh, 2003, Hyldahl et al., 2017). Changes in MTU behaviour between bouts of ECC exercise have been evidenced in recent years. Specifically, (Lau et al., 2015) found that there was a decrease in the amount of displacement of the muscle-tendon junction (MTJ) during the second bout of ECC exercise in the elbow flexors. Additionally, Penailillo et al. (2015) found that muscle fascicle lengthening was reduced in the *vastus lateralis* during a repeat bout of ECC cycling exercise. Both Lau et al. (2015) and Penailillo et al. (2015) reported a reduction in some, but not all, EIMD markers after the second bout of exercise and indicated that a change in tendon compliance might be the mechanistic driver behind the change in MTU behaviour, though neither study measured this. As mentioned earlier, MTU behaviour, particularly muscle fascicle lengthening, can influence the amount of EIMD suffered after a bout of ECC exercise (Hicks et al., 2017, Guilhem et al., 2016). Therefore, it would seem that if muscle fascicle lengthening is reduced upon exposure to a repeated bout of exercise, and a lower magnitude of EIMD is reported, the

behaviour of the MTU might mediate the RBE, though no study has measured both muscle and tendon behaviour independently over the two bouts of exercise.

Finally, there is evidence to suggest that variation in the magnitude of EIMD experienced differs between males and females (Wolf et al., 2012, Minahan et al., 2015, Joyce et al., 2014). A proposed explanation for this is the membrane stabilising effect that oestrogen might have (Tiidus, 2005, Kendall and Eston, 2002), as evidenced by lower levels of intramuscular proteins circulating in the blood in females, compared to males (Joyce et al., 2014, Enns and Tiidus, 2010). Another reported difference between males and females is the greater tendon compliance found in females (Onambele et al., 2007, Hansen and Kjaer, 2014) which can result in lower muscle fascicle lengthening over the same range of motion during ECC exercise (Hicks et al., 2013). This greater tendon compliance is thought to be due to the direct action of oestrogen on the tendon (Hansen and Kjaer, 2016), although oestrogen fluctuations across the menstrual cycle do not alter tendon compliance in the short term (Burgess et al., 2010). The use of oral contraceptive pill (OCP) supresses oestrogen concentrations throughout the menstrual cycle (van Heusden and Fauser, 2002, Elliott-Sale et al., 2013) leading to OCP using females reporting low oestrogen levels in comparison to eumenorrheic females (Bryant et al., 2008). Moreover, as oestrogen can directly act on tendinous tissues and might increase compliance (Hansen and Kjaer, 2016), the suppression of oestrogen in OCP using females might affect this action of oestrogen on tendons. However, there is mixed evidence for (Bryant et al., 2008) and against (Hansen et al., 2013, Burgess et al., 2009) differences in tendon properties between OCP using and eumenorrheic females. Nevertheless, given the relationship between MTU behaviour, EIMD and the RBE, there is precedent to further explore

the role that sex and OCP use might have in this area. Therefore, the aim of this thesis is to use US to determine if adaptations in the behaviour and properties of the *vastus lateralis* MTU influences the degree of EIMD and the protective effect conferred by a repeated bout of ECC exercise, and whether this differs between males, OCP using females, and eumenorrheic females.

CHAPTER 2 LITERATURE REVIEW

2-1 OVERVIEW

The objective of this literature review is to highlight the pertinent research that has led to the aims of each individual experimental chapter in this thesis, and the wider thesis as whole. The review begins with an overview of skeletal muscle structure and function, followed by a description of EIMD and regeneration, including how EIMD is measured and the potential determinants. The review then progresses to discuss the RBE and the mechanisms underpinning the phenomenon. The review finishes by summarising the evidence from studies that explore a potential sex difference in the EIMD and RBE response, with a focus on the role of reproductive hormones on skeletal muscle and tendon function. The collated evidence aims to provide a rationale for exploring the responses of the muscle tendon unit to repeat bouts of maximal eccentric exercise in males and females.

2-2 SKELETAL MUSCLE

Skeletal muscle is a highly organised structure of proteins accounting for approximately 40-45% of body mass (Snow, 2003), responsible for the production of joint movement and force exertion. Figure 2-1 illustrates the structure of the whole muscle to the myofibril, as currently accepted.

2-2.1 Skeletal Muscle Structure

A skeletal muscle consists of hundreds of muscle fibres arranged into bundles called fascicles (Jones et al., 2004). Each muscle fibre is surrounded by a selectively permeable membrane named the sarcolemma, which consists of a soluble layer of lipid molecules, predominantly phospholipids, with membrane proteins embedded within it (Widmaier et al., 2004). The sarcolemma encapsulates the water-based internal

environment of the muscle fibre termed the sarcoplasm, which hosts ions, enzymes, molecular gases and fuels required for muscle function (Woledge et al., 1985).

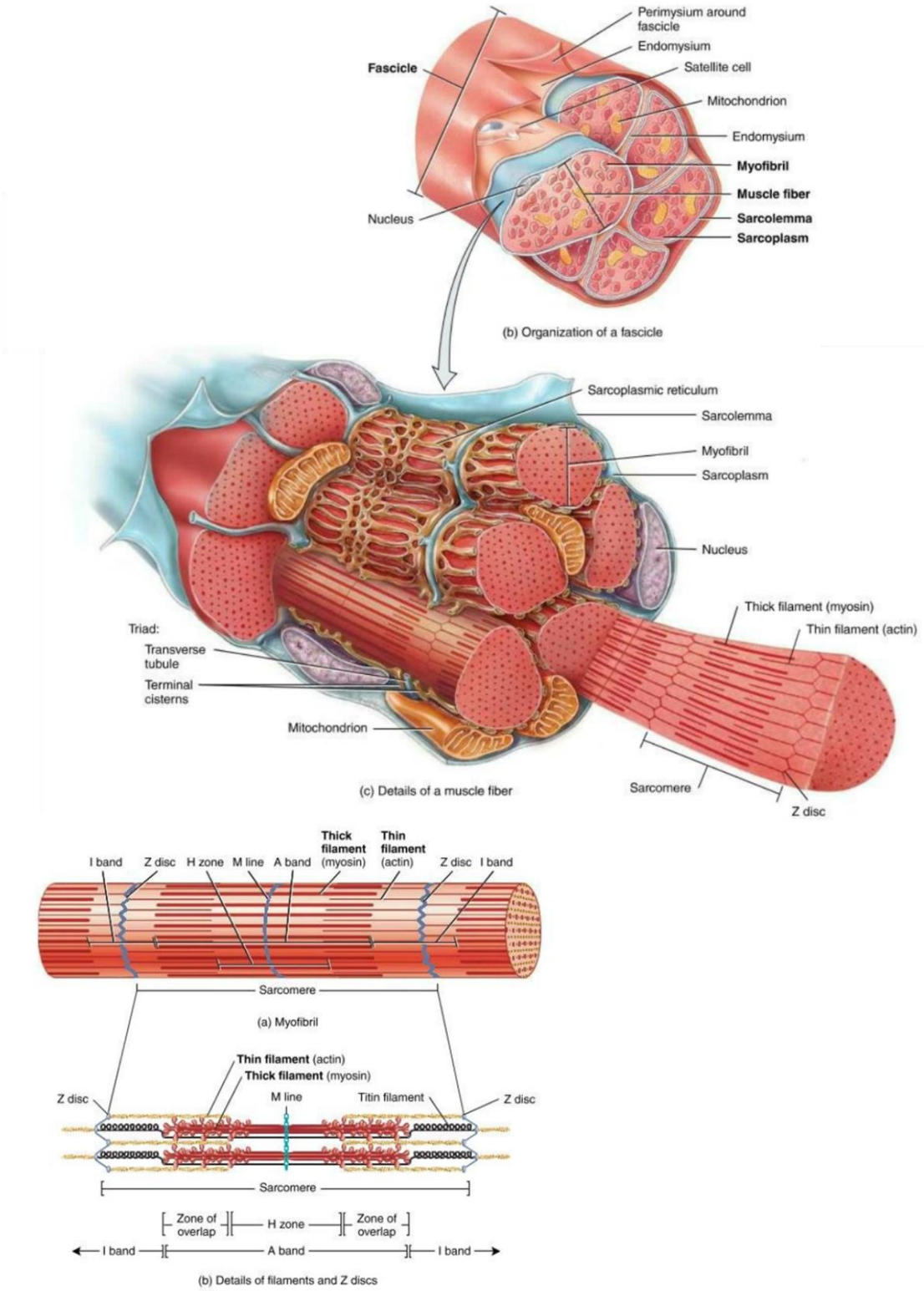

Figure 2-1 Structure and organisation of skeletal muscle (Tortora, 2017).

Each muscle fibre is made up of thousands of myofibrils, 1-2 µm diameter structures, which contain the contractile proteins that are responsible for force production (Woledge et al., 1985). Surrounding each myofibril is the internal system known as the sarcoplasmic reticulum (SR) which contains a highly concentrated store of CA^{2+}, contained in lateral sacs (McComas, 1996). The main function of the SR is to control the movement of CA^{2+} across the sarcolemma, to allow muscle contraction and relaxation to occur.

Myofibrils consist of hundreds of repeating units called sarcomeres, which contain the contractile proteins actin and myosin (Huxley, 1957), collectively constituting approximately 80% of skeletal muscle mass (Jones et al., 2004). The arrangement of these filaments is pictorially represented in Figure 2-1. Actin and myosin interact to produce the sliding of the filaments, with the actin bound proteins, tropomyosin and troponin, providing a controlling function (Woledge et al., 1985). The individual actin filaments are fixed in place to the Z-line by α-actinin, positioning them at the end of each sarcomere. The thick filaments, mainly consisting of myosin, are located in the middle of the sarcomere and joined to adjacent thick filaments by transverse connective tissue at the M-line. At the Z-line, thick filaments are attached via an elastic link provided by the spring like, giant structural protein, titin.

Figure 2-1 displays that titin connects to both the M-line and the Z-line, however only the I-band region of titin is functionally extensible via two, structurally distinct segments (Horowits and Podolsky, 1987). The first is the immunoglobulin (Ig)-like regions, arranged in tandem and located at the proximal and distal regions of the I-band. The second is the PEVK region, consisting of the four amino acids proline (P),

glutamate (E), valine (V) and lysine (K) (Labeit and Kolmerer, 1995). When exposed to stretch, the Ig-like segments lengthen first, followed by the PEVK regions, with the latter becoming the primary force-producing element (Trombitas et al., 1998). A second, large structural protein named nebulin contributes to the structural integrity to the sarcomere by strengthening the actin filaments (Jones et al., 2004).

A series of other cytoskeleton proteins including α-actinin, vinculin, talin, β_1 integrins and desmin (Patel and Lieber, 1997), form what is termed the costamere, which align circumferentially with the Z-disks in striated muscle, coupling the sarcolemma to the force generating sarcomeres (Ervasti, 2003). In addition to contributing to the structural integrity of the myofiber, costameres might function to laterally transmit force to adjacent muscle fibres, maintaining consistent sarcomere lengths between contracting and relaxing muscle cells (Street, 1983, Danowski et al., 1992, Craig and Pardo, 1983). Finally, in addition to the contributing to costamere formation, desmin is a major component of intermediate filaments that act as a mechanical stabiliser of the myofiber (Lazarides, 1980), which acts to limit extreme sarcomere lengths (Wang and Ramirez-Mitchell, 1983). For optimal muscle function to occur, the integrity of these structural proteins must be intact. Therefore, the loss or damage of one or more of these proteins can lead to impaired muscle performance, such that is seen as a result of EIMD.

2-3 SKELETAL MUSCLE FUNCTION

2-3.1 Sliding filament theory

The theory that the thick and thin, myosin and actin filaments in skeletal muscle 'slide' over each other to produce force was developed in the early 1950's and termed the

'sliding filament theory' (Huxley, 1957). This was evidenced using light (Huxley and Hanson, 1954, Huxley and Niedergerke, 1954) and electron (Page and Huxley 1963) microscopy that showed that the length of the individual filaments did not change, despite the shortening of the muscle fibre. It is widely accepted that the sliding movement of the filaments is produced via the formation of cross-bridges between the actin filament and the myosin head (Woledge et al., 1985). Although cross-bridge kinetics are beyond the scope of this thesis, in brief, actin, myosin and adenosine triphosphate (ATP) interact to produce cross-bridge movement and subsequent force production, with the requirement of ATP hydrolysis to detach the cross-bridge and repeat the cyclical process (Huxley and Simmons, 1971, Huxley, 1957). One observation of the cross-bridge theory is that the ATP cost for lengthening (eccentric) muscle actions is lower than that for constant-length (isometric) and shortening (concentric) muscle actions (Abbott et al., 1952), which will be discussed further in section 2-3.3.

Cross-bridges occur via a sequence of events termed excitation-contraction (E-C) coupling, which is initiated via a signal form the central nervous system that stimulates the muscle. This sequence of events involves (1) the initiation and propagation of an action potential along the sarcolemma, (2) The spread of the potential along the transverse-tubule system, (3) dihydropyridine receptors (DHPR)-mediated detection of changes in membrane potential, (4) interaction of DHPR with ryanodine receptors in the SR, (5) release of Ca^{2+} from the SR and transient increase of Ca^{2+} in the sarcoplasm, (6) Ca^{2+} interacts with troponin-tropomyosin complex, removing the inhibitory effect of troponin I and tropomyosin, allowing the actin-myosin interaction to occur and the thin filaments to slide over the thick ones, thus producing tension,

followed by (7) the reuptake of Ca^{2+} from sarcoplasm, mainly by the reuptake by the SR through the SR Ca^{2+} adenosine triphosphatase, leading to the removal of tension and allowing for subsequent muscle relaxation (Sandow, 1965, Fill and Copello, 2002, Calderon et al., 2014). In healthy, undamaged muscle, this process will repeat until there is no longer a requirement for contraction. However, damage to one or more elements of the muscle contraction process could impair the ability of the muscle to contract and produce force. It is therefore important to understand the effect that damaging exercise can have on the muscle contraction process and this is discussed in section 2.4.

2-3.2 Length-tension relationship

The force produced by muscle fibres is widely accepted to be proportionate to the length of the sarcomere and the degree of actin and myosin overlap (Gordon et al., 1966). This is known as the length-tension (LT) relationship and is demonstrated in Figure 2-2. However, it must be noted that the model by Gordon et al., (1966) is based on isolated muscle and *in vivo*, the skeletal muscle length-tension relationship typically operates in a narrower range, and can be affected by changes in passive tension throughout the contraction (Hoffman et al., 2014). This will be discussed in more detail below.

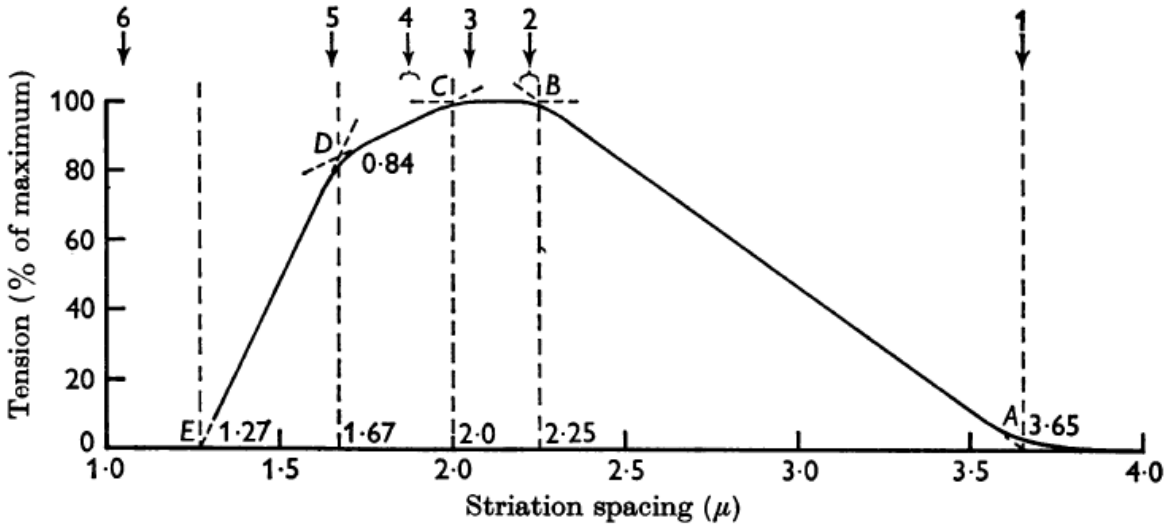

Figure 2-2 Gordon's length-tension relationship (Gordon et al., 1966).

In Figure 2-2 at point C, the maximum amount of tension is produced due to maximum myosin-actin filament overlap, with tension plateauing between points B and C, due to no cross-bridge sites being added. At point D (short sarcomere lengths) the thick filaments collide with the Z-disks and tension decreases rapidly. At this point, Gordon et al., (1966) stated that tension is also reduced at short sarcomere lengths due to the thin filaments interfering with one another. At lengths beyond point B (lengthening actions), there is a linear reduction in tension. On this descending limb of the LT relationship, the sliding filament theory cannot explain the amount of tension produced at long sarcomere lengths. If the only factor determining the LT relationship was the number of cross-bridges formed, the LT curve would be U-shaped, with tension equal to cross-bridge formation either side of the plateau (points B to C). Herzog (2014) proposed that the passive force produced by titin during sarcomere lengthening can account for the slower decline in tension on the LT curve.

In the original LT relationship study, Gordon et al. (1966) was unable to achieve a steady force state at maximum tension at a given sarcomere length, known as the

"creep" phase of contraction. The authors therefore used force extrapolation before reaching maximum tension to avoid the creep phase and provided the LT relationship described in Figure 2-2, with several other studies showing a similar response using this method (Granzier and Pollack, 1990, Edman and Reggiani, 1987, Bagni et al., 1988). These studies, in addition to using extrapolated forces, allowed sarcomeres to shorten freely. Several other studies that measured maximal forces whilst clamping a portion of the sarcomeres produced contrasting results, which can be seen in Figure 2-3 (Terkeurs et al., 1978, Martyn and Gordon, 1988, Fabiato and Fabiato, 1978, Endo, 1972, Close, 1972, Carlsen et al., 1961).

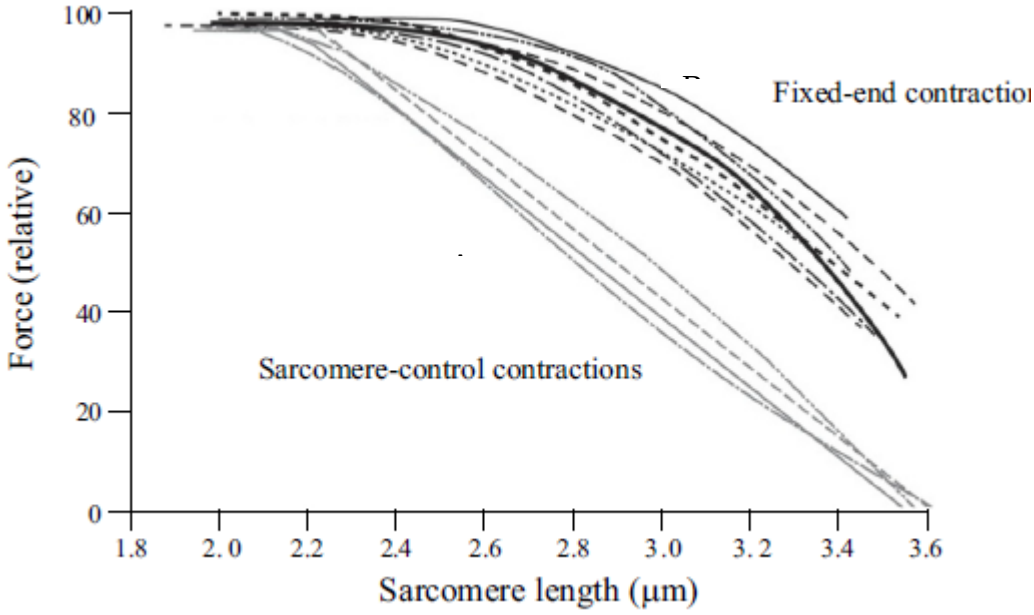

Figure 2-3 Length tension relationship without (b) and with (a) sarcomere length clamping. Adapted from Pollack (1983).

These studies found that there was little force variation between maximum thick/thin filament overlap and 50% overlap, and forces of ~20% at sarcomere lengths of 3.8 µm - 4.0 µm, where no force should be produced according to the LT relationship reported by Gordon (1966). The differences in LT relationship, depending on sarcomere

clamping, can been attributed to sarcomere length non-uniformity which develops when fibres lengthen after they have been allowed to shorten first (Rassier, 2017). This theory is supported by evidence showing that proximal sarcomeres in the fibre shorten during contractions at long muscle lengths, whilst more central sarcomeres lengthen (Edman and Reggiani, 1987, Edman and Reggiani, 1984). The forces produced by the fibre will be closer to isometric strength of the shorter and stronger sarcomeres, rather than the longer sarcomeres, due to the elongating side of the force-velocity curve being steeper than the shortening side (Morgan et al., 1982, Morgan et al., 1991). This could mean that the lengthened (weaker) sarcomeres could be vulnerable to "popping" on the descending limb of the LT curve, as suggested by Morgan (1990), however there is evidence to suggest that sarcomeres are stable at different lengths of the LT descending limb. Specifically, following the active stretching of single myofibrils onto the descending limb of the LT curve, Rassier et al. (2003) reported that the anticipated over-stretching of the actin-myosin overlap did not occur, and that sarcomeres were stable at all recorded lengths of the LT curve. Given the mixture of evidence of sarcomere stability at different lengths, a comprehensive and satisfactory explanation for the observation of increased force on the descending limb of the LT relationship is still needed.

Several other factors can affect the LT relationship. For example, lower muscle activation produces maximal force generation at longer muscle lengths (Stephenson and Wendt, 1984, Rack and Westbury, 1969, Close, 1972, Balnave and Allen, 1996), attributed to a length dependence of calcium sensitivity (Stephenson and Wendt, 1984, Rassier et al., 1999). The LT relationship has also been shown to be related to changes in myofilament lattice spacing (Fuchs and Smith, 2001, MacIntosh, 2017, Rassier et

al., 1999, Williams et al., 2013, Yang et al., 1998), thick and thin filament regulation (de Tombe et al., 2010), and the effect of Ca^{2+} sensitive sarcomeric regulatory proteins at the actin-myosin binding site (de Tombe et al., 2010). The LT relationship is an extremely important concept to understand in the context of this thesis, as it is part of the underlying theory of EIMD. The relevance of the LT relationship to ECC muscle action will be discussed in section 2-3.4.

2-3.3 The in vivo application of the popping sarcomere theory

The evidence pertaining to the popping sarcomere theory presented in the previous section is largely based on single muscle fibre studies *in situ* or in *vitro*, and therefore cannot be directly extrapolated to *in vivo* applications. For example, Hoffman et al. (2014) found that during backwards downhill walking, gastrocnemius fascicles predominantly contracted to the ascending and plateau regions of the LT curve, despite losses in iMVC torque following exercise. Previous literature has suggested that the role of the tendon *in vivo* might be responsible for this altered fascicle behaviour (Butterfield and Herzog, 2005, Austin et al., 2010), something that single fibre *in situ* and *in vitro* studies does not account for. This lack of tendon interaction during *in vitro* and *in situ* studies can result in fascicle lengthening of up to 125% (Lieber & Friden, 1993) versus *in vivo* studies, where fascicle lengthening of ~18% in the gastrocnemius (Hoffman et al., 2014) and 58% and 40% in the VL for males and females, respectively (Hicks et al., 2013), can occur. The popping sarcomere theory remains an important and plausible explanation for the early stages of EIMD, however, there is a lack of *in vivo* evidence to support this and therefore the extrapolation of findings from *in vitro* and *in situ* research should be made with caution.

2-3.4 Skeletal muscle actions

The action of skeletal muscle provides the force required to initiate the movement of joints, however not all muscle actions are mechanistically equal. In comparison to concentric (CON) or isometric (ISO), ECC muscle actions produce specific adaptations that might be explained by the unique features of the ECC action (Duchateau and Baudry, 2014, Guilhem et al., 2010). In comparison to CON contractions, ECC muscle actions require less motor unit activation and consume less oxygen and use less energy at a given force (Abbott et al., 1952), whilst producing greater forces for a given angular velocity (Hortobágyi and Katch, 1990). Although not fully understood, it is generally well accepted that there are considerably different neural strategies for controlling ECC muscle actions, when compared to CON and ISO contractions (Duchateau and Baudry, 2014). These differences are evidenced at the cortical level, as well as the contracting muscle, with research suggesting that, when compared to CON and ISO actions, reduced central activation is present during ECC muscle actions, which has implications for fine motor control during eccentric actions (Hoppeler and Herzog, 2014). Further unique characteristics of ECC muscle actions include a greater cortical excitability but a lower motor unit discharge rate, and a greater voluntary deficit compared to CON contractions when assessed using the twitch interpolation technique, such that untrained individuals are usually unable to fully activate their muscles during maximal eccentric muscle actions (Hoppeler and Herzog, 2014). Collectively, the unique qualities of ECC muscle actions that lead to increase force production are not well understood, but there are several theories that might provide an explanation.

The original cross-bridge theory (Huxley, 1957) cannot explain the increased force production encountered during ECC muscle actions for several reasons. It is assumed that each cross-bridge has the same force-potential and that they are aligned uniformly along the myosin filament (Huxley, 1957). The point at which the thick/thin filament overlap is equal on the ascending and descending side of the length-tension relationship should, according to the cross-bridge theory, produce equal force, however this is not the case. Additionally, the cross-bridge theory suggests that the hydrolysis of 1 ATP molecule is required for the detachment of the myosin head to the actin binding site for CON and ISO contractions (Huxley, 1957). Huxley (1957) realised that this energy cost did not fit with ECC muscle actions and suggested that multiple cross-bridge sites per ATP molecule might be possible. Other explanations for the low energy cost of ECC muscle actions include that energy is dissipated rather than produced (Ryschon et al., 1997) and actin and myosin bonds 'breaking' mechanically rather than requiring ATP hydrolysis (Huxley 1957, Ryschon et al., 1997), with the latter potentially contributing to the increased amount of EIMD experienced following ECC muscle actions. Although there is no one theory that can explain the low energy cost of ECC contractions, it is clear that they are unique in nature. It has been suggested that structural components within skeletal muscle might have an effect on the low energy cost of ECC contractions (Herzog, 2014). This interplay between muscle structure and action will be discussed in the next section.

2-3.5 Kinetics of active muscle lengthening

An actively lengthened muscle fibre can produce an increased amount of force at a given length in comparison to an isometric control, a discovery made by Edman et al., (1982) and termed residual force enhancement (RFE), which was later supported by

Herzog et al. (2006). Not only did the actively stretched fibre produce more force, it was done with a lower amount of ATPase activity in compared to a non-stretched fibre, indicating that fibres become more efficient after stretch (Joumaa and Herzog, 2013), which makes the mechanism behind RFE an excellent fit to explain the unique contractile behaviour of ECC muscle actions. Herzog and Leonard (2002) tested this hypothesis on the cat soleus muscle and concluded that when actively stretched, passive structural elements engaged, producing increased passive force which persisted for minutes following activation, but the force enhancement disappeared quickly if the muscle was returned to starting length. The engagement of the structural protein titin was demonstrated to be responsible for this passive force enhancement. This was confirmed when single myofibrils showed the increase in passive force after active lengthening but did not show this increase when titin was removed in the sarcomeres of the myofibril (Leonard et al., 2010).

The engagement of titin during muscle action has been proposed to occur in two ways: (1) titin binds with calcium upon activation, increasing its inherent stiffness in the PEVK (Labeit et al., 2003) and Ig regions (DuVall et al., 2013), and (2) titin can bind proximally to actin, thus shortening the active length of its spring (Herzog, 2018), as demonstrated in Figure 2-4. Ca^{2+} binding to titin only accounts for 10-20% of the increased force observed during and after ECC muscle action (Leonard and Herzog, 2010). Using fluorescently labelled titin antibodies, it was observed that in passive stretching, both the proximal and distal segments of titin elongated. However, in active stretching (presence of Ca^{2+}) there was reduced lengthening of titin in the proximal region, whereas the distal region continued to lengthen, with the authors interpreting that the proximal region of titin bound with actin, therefore reducing lengthening

capacity and increasing stiffness and subsequent force production (DuVall et al., 2017). Although still in its infancy, this theory of the adaptability of titin could explain the higher force producing and more energy efficient attributes of ECC muscle actions based on titin's effect on muscle stiffness.

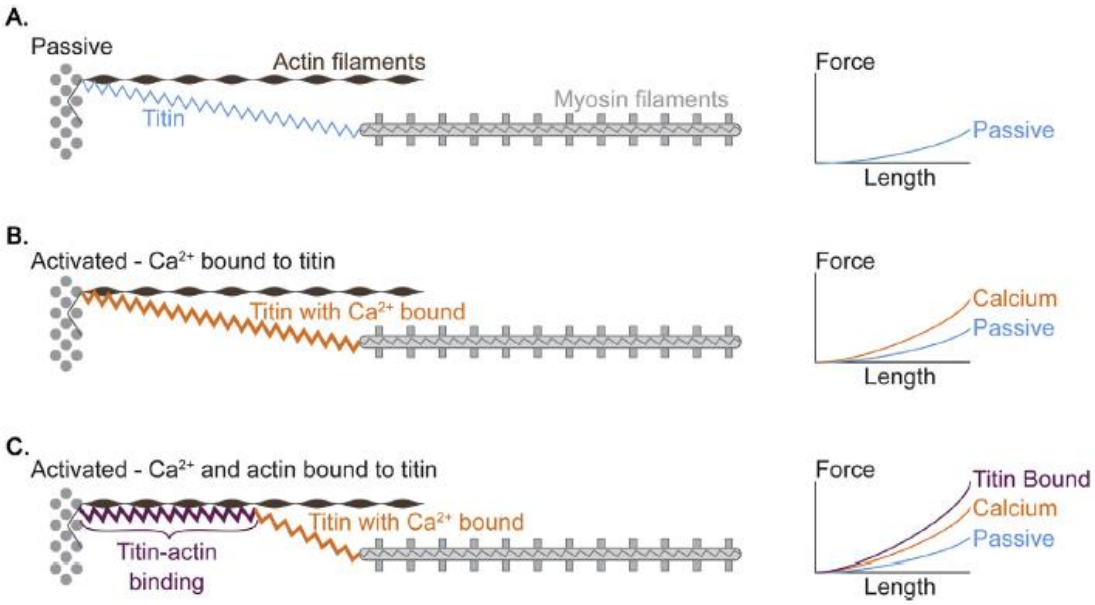

Figure 2-4 The mechanics of titin in passive (A) and active (B, C) sarcomeres. (A) passive stretching elongates the entire I-band region of titin. (B) the presence of calcium in active lengthening increases titin's stiffness. (C) Titin binds to actin upon activation and stretching, reducing the free spring length (Herzog, 2018).

2-4 EXERCISE INDUCED MUSCLE DAMAGE

Following strenuous exercise, it is common for humans to experience a range of symptoms that include a loss of muscle function, soreness, swelling, a decreased range of motion and leakage of muscle proteins into the blood occurring both immediately and for up to ~14 days after exercise (Clarkson et al., 1992, Proske and Allen, 2005). This collection of symptoms is commonly termed exercise-induced muscle damage. Eccentric muscle actions produce significantly higher levels of EIMD in comparison

to CON and ISO muscle contractions (Penailillo et al., 2013, Willoughby and Taylor, 2004), which is thought to be due to the unique contractile mechanisms of ECC muscle actions, as discussed in the previous section, leading to greater amounts of total force (or torque), particularly at long muscle lengths (Guilhem et al., 2016, Lieber and Friden, 1993, Nosaka et al., 2005a, Penailillo et al., 2013, Penailillo et al., 2015). Although other mechanistic properties of ECC muscle actions are thought to be involved in the increased levels of EIMD, the greater levels of force during active muscle lengthening in comparison to ISO or CON muscle actions, are the primary focus of this review.

Typically, EIMD is characterised by a multitude of symptoms that are present both immediately after exercise and can last up to 14 days, depending on the individual's susceptibility to the damaging stimulus (Douglas et al., 2017). Consequences of EIMD include a reduction in functional capacity, increased muscle soreness (Proske and Morgan, 2001), disturbed sense of force production and limb position (Paschalis et al., 2010) and reductions in exercise capacity (Marcora and Staiano, 2010). Exercise induced muscle damage can be identified via observations of direct adjustments in the muscle apparatus, such as sarcomere disruption and EC coupling failure, or via indirect adjustments, such as loss of muscle function, soreness, and the presence of intramuscular proteins in the blood (Hyldahl and Hubal, 2014, Clarkson and Hubal, 2002). Armstrong (1990, 1991) proposed that the pathologies associated with EIMD occur as part of a four-stage process (1) initial stage, (2) loss of Ca^{2+} homeostasis / autogenic phase, (3) phagocytic phase and (4) regenerative stage. For the purposes of this thesis, the four stages promoted by Armstrong (1990) will be combined to describe primary and secondary muscle damage.

2-4.1 Primary muscle damage

2-4.1.1 The popping sarcomere theory

Eccentric muscle actions, as aforementioned, require different neural strategies to control muscle force in comparison to CON and ISO actions (Duchateau and Baudry, 2014). In addition to this, ECC muscle actions have a lower motor unit recruitment for a given force in comparison to CON and ISO contractions (McHugh et al., 2000) meaning that a smaller number of fibres experience greater mechanical stress (Enoka, 1996). It is generally well accepted that the initial stage of muscle damage is orchestrated by asymmetrical lengthening of sarcomeres, leading to high levels of mechanical stress, mechanical overload and subsequent damage. This theory, known as the 'popping sarcomere theory' (Morgan, 1990) suggests that the weakest sarcomeres in the fibre absorb most of the length change over the range of motion, beyond the point of myofilament overlap, resulting in a 'popping' of the sarcomere and an increase in tension on the passive structures. Upon relaxation, some weakened sarcomeres fail to re-interdigitate and become disrupted (Talbot and Morgan, 1996). These weaker and disrupted sarcomeres might lead to the shearing of myofibrils, leading to a loss of calcium homeostasis and subsequent EIMD, due to the deformation of membranes and t-tubules. The contribution of a loss of calcium ion homeostasis to EIMD will be discussed further in the next section. Morgan (1990) suggested that as a result of fibres failing to re-interdigitate, repeated active lengthening would place extra tension on neighbouring sarcomeres, resulting in "tearing" of those sarcomeres. This could inevitably result in the amount of fibre disruption increasing as muscle actions are repeated (Proske and Allen, 2005). The popping sarcomere theory is supported by evidence suggesting that the length of the muscle during ECC muscle

action (Talbot and Morgan, 1998, Patel et al., 2004, Lieber and Friden, 1993) and number of contractions (Hyldahl and Hubal, 2014), are key factors in determining the extent of EIMD following ECC exercise. However, as discussed earlier in section 2-3.3, the *in vivo* evidence supporting the popping sarcomere theory is weak and therefore its application should be used cautiously.

2-4.1.2 Failure of the excitation-contraction coupling process

An alternative theory proposed by Warren et al. (2002) suggests that a failure of the E-C coupling process is the primary event initiating EIMD. This theory was originally constructed by Warren et al. (1993b) after investigating the use of caffeine to stimulate Ca^{2+} release from mouse muscle fibres. It was found that by triggering the release of Ca^{2+} from the SR, the decrement of force loss following twenty ECC contractions was negated (Warren et al., 1993b). Corona et al. (2010) further explored E-C coupling failure and EIMD by examining the muscle damage response to fifty ECC contractions in mice. Following exercise there was a reduction of tetanic isometric force immediately post exercise, day one and day three vs baseline (70.7 ± 1.6, 77.4 ± 3.1 and 89.2 ± 3.9 vs 111.9 ± 2.5 $N \cdot mm^{-1} \cdot kg^{-1}$ *(*$P < 0.05$*)*, respectively). Corona et al. (2010) then compared caffeine contracture and peak tetanic force deficits following the ECC exercise and estimated that ~49%, 58% and 45% of the force deficits observed at immediately post exercise, day one and day three, respectively, was attributable to E-C coupling failure. Moreover, it was then concluded that the E-C coupling failure was associated with the reduction of junctophilins 1 (~50% reduction) and 2 (35% reduction), which are the primary proteins involved in T-tubule and SR membrane apposition (Komazaki et al., 2002, Ito et al., 2001, Takeshima et al., 2000).

Although Corona et al. (2010) stated that the role of other triadic proteins require further investigation, their evidence suggests that junctophilin damage might account for, in part, the early force deficit due to E-C coupling failure.

The failure of E-C coupling can also be evidenced by the measurement of Low-frequency fatigue (LFF), which is characterised by reductions in forces at low (10-20 Hz) compared to higher (50-100 Hz) electromyostimulation frequencies (Jones, 1996, Clarkson and Hubal, 2002). It has been suggested that these force losses at low stimulation intensities can be attributed to reduced Ca^{2+} release form the SR, indicating E-C coupling impairment (Dundon et al., 2008). Although, Jones (1996) suggested that in addition to reductions in Ca^{2+} release, structural damage to the myofibril might contribute to LFF, based on observations of the force-frequency relationship being length dependent. Jones (1996) stated that if the popping sarcomere theory was correct and the central sarcomeres in the fibre "popped" while the total fibre length remained the same (Morgan, 1990), the force-frequency curve following damaging exercise would shift to the right, as it would if the muscle was placed in a shorter position. This was indeed evidenced by Child et al. (1998), who observed a shift to the right in the L-T relationship, with the greatest loss in force evident when tested at the shortest muscle length. Nevertheless, the actual contribution of Ca^{2+} release or myofibril structural damage to LFF remains unclear but it remains evident that LFF is due, in part, to a deficit in E-C coupling (Muanjai et al., 2020). The theory of E-C coupling failure as a mechanism of primary muscle damage, proposed by Warren (1993) has been challenged by Proske and Allen (2005), stating that E-C coupling failure was a consequence of sarcomere damage, not an initiating event. Although there are contrasting arguments to explain the initial events of EIMD, it is generally accepted

that ECC muscle action disrupts both the contractile and non-contractile elements and the E-C coupling process, suggesting that both mechanisms are likely to contribute to the manifestation of EIMD (Hyldahl and Hubal, 2014). Ingalis et al. (1998) stated that over the first three days following damaging exercise, 57-75% can be ascribed to the impairment of the E-C coupling process, with the remainder attributed to contractile apparatus damage in the first one to two days, followed by decreased contractile protein content in the days following. Whilst the exact proportion that E-C coupling failure plays in the loss of strength following damaging exercise is debatable, it is clear that strength loss following ECC exercise is derived from a complex interplay between various mechanisms that require further clarification, particularly in human studies.

2-4.1.3 Metabolic Damage

Another theory to explain the initial cause of EIMD is the metabolic stress experienced during exercise (Tee et al., 2007). Exercise that has involved low levels of ECC muscle action but high levels of metabolic stress, such as repeated sprint cycling (Rankin et al., 2018), has reportedly increased markers of EIMD (discussed in detail in section 2-5), such as increased serum creatine kinase (CK and a reduction in force production, although the change in these markers are modest (Saunders et al., 2004, Rankin et al., 2018) and the mechanism of this result is not well understood. Within the model of metabolic stress and EIMD by Tee et al. (2007), there are theories pertaining to EIMD: insufficient mitochondrial respiration, the production of reactive oxygen and nitrogen species (RONS) and high intramuscular temperatures. These theories are however, beyond the scope of this thesis and the interested reader is referred to Tee et al. (2007).

2-4.2 Secondary Muscle damage

The initial events of EIMD trigger several events that lead to a more severe, secondary damage to the muscle. These are categorised into three main processes, (1) The disturbance of Ca^{2+} homeostasis, (2) The inflammatory response to EIMD, and (3) Oxidative stress.

2-4.2.1 – Disturbance of calcium homeostasis

Following the primary events of EIMD there is uncontrolled movement of Ca^{2+} into the cytoplasm that might cause further damage (Ebbeling and Clarkson, 1989, Armstrong, 1984), which is a potential consequence of membrane disruption (Lieber and Friden, 2002) or the opening of stretch activated channels (Overgaard et al., 2002). This movement results in abnormally high levels of intracellular Ca^{2+} which can result in the degradation of structural proteins, following the activation of Ca^{2+} dependant proteolytic and phospholipase A2 pathways (Gissel and Clausen, 2001, Gissel, 2005). Specifically, these proteases, named calpains, are suggested to contribute to EIMD, as they are responsible for cleaving the proteins in charge of myofibril integrity (desmin and alpha-actinin) (Zhang et al., 2008), with these structural proteins being further degraded by other proteolytic pathways such as the ubiquitin-proteasome system (Raastad et al., 2010). In addition, increased entry of Ca^{2+} into the cell might also occur as a result of the degradation of membrane components, resulting from the activation of calpains (Raastad et al., 2010).

In a normal intracellular environment, mitochondria help maintain Ca^{2+} homeostasis by excess uptake (Ebbeling and Clarkson, 1989, Gissel, 2005). However, Ca^{2+} overload following EIMD can alter mitochondrial respiration (Rattray et al., 2013) and

lead to the permeabilisation of the inner mitochondrial membrane followed by the opening of the mitochondrial permeability transition pore (Gissel, 2005). This in tum, can cause a large influx of intracellular Ca^{2+} from the mitochondria, and a further increase in calpain proteolytic activity, resulting in mitochondrial dysfunction, apoptosis and necrosis (Gissel, 2005). Further consequences of increased intracellular Ca^{2+} include uncontrolled muscle contraction, which might contribute to the increases in passive forces observed following EIMD (Proske and Morgan, 2001, Proske and Allen, 2005, Allen, 2001). Furthermore, increased calpain activity can promote the activation of neutrophils and macrophages, eventually leading to the production of RONS (Powers and Jackson, 2008). The process of secondary muscle damage is thought to be predominantly instigated by the aforementioned inflammatory response and will be discussed below.

2-4.2.2 – Inflammatory Response

The inflammatory response to EIMD is a dynamic, co-ordinated process that is necessary to produce adaptive remodelling of muscle tissue and an eventual retum to homeostasis (Chazaud, 2016). This process is primarily co-ordinated by intracellular signalling molecules labelled cytokines (Cannon and St Pierre, 1998). Cytokines regulate the inflammatory process, which begins with the accumulation of leukocytes (neutrophils, macrophages and t-lymphocytes) in the damaged muscle tissue, evidenced by histological observations following muscle damaging exercise in humans (Paulsen et al., 2012). Neutrophils are the first leukocyte group to infiltrate the damaged tissue, evidenced by elevated circulating neutrophils between 2-12 hours following EIMD (Suzuki, 2018, Pizza et al., 1995, Kawanishi et al., 2016). It is

thought that the initial function of neutrophils is a phagocytic action (Chazaud, 2016, Butterfield and Herzog, 2006), achieved by the deployment of proteolytic and cytotoxic molecules that phagocytose necrotic myofibers and cellular debris (Pizza et al., 2005). Neutrophils also secrete a number of pro-inflammatory cytokines, which attract an increased number of neutrophils to the damaged tissue via a positive feedback loop that magnifies the inflammatory response (Butterfield and Herzog, 2006). This intensified local immune response can aggravate existing damage and has implicated neutrophils in the secondary damage process (Nguyen and Tidball, 2003), which has been evidenced by further loss of muscle function in subsequent days following EIMD (Toumi and Best, 2003, Howatson and van Someren, 2008), although this is not always the case (Warren et al., 2017).

The second group of leukocytes that play a role in the inflammatory response to EIMD are macrophages, which like neutrophils, produce cytotoxic molecules that contribute to the degradation of necrotic tissue (Nguyen and Tidball, 2003). However, unlike neutrophils, macrophages produce several growth factors that support tissue remodelling (Tidball, 2005). In addition, macrophages also have three distinct populations (ED1+, ED2+ and ED3+), that are activated sequentially, suggesting multiple different functions (Smith et al., 2008, Butterfield and Herzog, 2006). ED1+ macrophages appear within damaged muscle fibre within 24 hours of EIMD and have phagocytic functions, promoting the removal of damaged tissue (Butterfield and Herzog, 2006), and pro-inflammatory functions, via the attraction of additional neutrophils, further exacerbating the inflammatory response (Kharraz et al., 2013). ED2+ and ED3+ macrophage groups are not thought to be involved in the phagocytic stage of the immune response (Smith et al., 2008), but instead might promote tissue

regeneration through the promotion of growth factors such as insulin-like growth factor, transforming growth factor beta, and fibroblast growth factor, all of which are necessary for tissue regeneration and growth (Urso, 2013, Smith et al., 2008). It is clear from the available evidence that there is a targeted immune response from predominantly neutrophils and macrophages, however the distinct role of each leukocyte is unclear and it is likely that the four stage model proposed by Armstrong (1990) overlaps at several stages.

2-4.2.3 – Oxidative Stress

Intense exercise can produce excess RONS that can shift the metabolic environment out of homeostasis into a state of oxidative stress (Nikolaidis et al., 2008, Kohen and Nyska, 2002), which can lead to molecular damage and a reduction in redox signalling (Sies, 2017). Specifically, intense ECC based muscle damaging protocols have been shown to produce a higher amount of RONS than CON or ISO contractions, with levels peaking between 24-96 hours (Nikolaidis et al., 2008), most likely due to the increased inflammatory response associated with ECC induced EIMD (Nikolaidis et al., 2012, Nikolaidis et al., 2008). There is evidence to suggest that this increased production of RONS, resulting from ECC muscle actions, might contribute to secondary muscle damage, leading to a further increase in muscle soreness (Zerba et al., 1990) and an impairment of muscle function (McArdle et al., 1999), albeit this evidence is based on animal studies. Nevertheless, it is possible that if RONS remain within specific parameters following exercise, an endogenous antioxidant response can maintain homeostasis without performance being affected (Mello et al., 2017), however this protective mechanism is insufficient if RONS levels are too great and tip

the balance into an oxidative state, subsequently compromising exercise performance (Powers and Jackson, 2008). Therefore, effective endogenous antioxidant compounds, such as nutritional and pharmacological interventions (Howatson and van Someren, 2008, Owens et al., 2018) might play a role in mediating the potential for RONS to elicit secondary muscle damage.

2-4.3 Regenerative phase

Skeletal muscle has an excellent regenerative capacity that is evident on a daily basis and in response to exercise induced damage (Doyonnas et al., 2004). The process of muscle regeneration is a highly co-ordinated process that is dependent on the balance between pro-inflammatory and anti-inflammatory factors, that ultimately mediate repair (Prisk and Huard, 2003, Mourkioti and Rosenthal, 2005).

Following EIMD, the repair process is initiated by the subsequent inflammatory response that was discussed in section 2-4.2. Following the co-ordinated inflammatory response, after the initial stage of injury and the removal of cellular debris, certain cytosolic structures and disrupted myofilaments, macrophages also activate mononuclear myogenic cells, commonly known as satellite cells, in preparation for tissue regeneration (Lescaudron et al., 1999). When not exposed to a stressed condition, satellite cells remain quiescent, but once activated, proliferate, and differentiate to contribute to the repair of damaged fibres, as well as forming new myofibers (Hirata et al., 2003). This process of satellite cell fusion is possible through the creation of intracellular junctions, which contribute to the intracellular cytoskeleton, through the action of such molecules as M-cadherin and M-Calpain (Karalaki et al., 2009). M-cadherin, a calcium dependent intracellular adhesion

molecule, is expressed in skeletal muscle cells and myoblasts in response to injury and plays an important role in the fusion of myoblasts into myotubes (Kaufmann et al., 1999). M-calpain is a nonlysosomal cysteine protease which is more abundant during myoblast fusion (Dedieu et al., 2002) and produces the modification of cytoskeleton and membrane organisation during myoblast fusion (Dourdin et al., 1999). Once the muscle fibres are repaired, the satellite cell pool is restored and remains quiescent until required, giving the muscle the ability to repeatedly and completely regenerate (Karalaki et al., 2009). However, myogenic satellite cells are only one part of the muscle tissue regeneration process.

The extracellular matrix was originally thought to act as a scaffold for the arrangement of cells between tissues, playing only a passive role in muscle regeneration (Karalaki et al., 2009). However, elements that form part of the ECM, such as proteoglycans and collagen, might play a key energetic role in muscle development (Midwood et al., 2004, Osses and Brandan, 2002), although the role of these ECM components in muscle regeneration are not fully understood. What has been observed is that satellite cells are surrounded by ECM components, with evidence of the regeneration of these ECM molecules during migration, fusion and maturation of the myotubes, suggesting a relationship between the satellite cells and ECM components (Lewis et al., 2000, Carmeli et al., 2004, Guérin and Holland, 1995). Additionally, upon degradation, the ECM releases a number of growth factors such as decorin, that can upregulate the muscle tissue regeneration process (Hirata et al., 2003, Koutsilieris et al., 1997). Finally, the key component of the ECM, collagen, is synthesised as type III collagen following the initial stages of EIMD, with the synthesis of type I collagen increasing after approximately one week (Lehto et al., 1985). This collagen remodelling might

play a key role in the adaptive process of the muscle in response to EIMD by increasing the structural integrity of the muscle and more efficiently transmitting lateral force (Grounds et al., 2005), subsequently protecting the muscle from repeated bouts of similar exercise (Takagi et al., 2016), which is discussed in detail in section 2-7.2.3.

The regeneration process of muscle tissue and the contribution of the ECM to this process is not fully understood. However, it is clear that there are both individual and integrated roles of each component, north of which might mediate the extent of the regeneration and adaption process.

2-5 MARKERS OF EXERCISE INDUCED MUSCLE DAMAGE

The effects of EIMD are predominantly reported at the sub-cellular level, however quantifying the magnitude of this effect is often done via both direct and indirect measures (Warren et al., 1999). Direct measures require collection of muscle tissue in the area(s) of interest, whereas indirect measures are less invasive (with the exception of blood sampling) and typically encompass the collection of data pertaining to changes in muscle function, muscle soreness, limb girth, or intramuscular proteins, inflammatory, or oxidative stress markers in the circulation. Direct measures of EIMD can be expensive and difficult to measure, therefore, indirect measures of EIMD are used by many researchers. The subsequent sections will discuss each type of measurement method and how they have been commonly used to quantify the magnitude of EIMD experienced following damaging exercise.

2-5.1 Direct markers of exercise induced muscle damage

The use of muscle biopsies has commonly been used to measure damage to the structural components of the muscle. Typically, muscle samples of 10-50 g are used

for ultrastructural and histochemical analysis (Friden et al., 1983), with evidence suggesting that histochemical abnormalities are present 12 hours post-exercise, with peak muscle damage occurring at 48 hours (Yasuda et al., 1997). The use of muscle biopsies has been used to determine structural damage to both the sarcomere and the E-C coupling apparatus, which will be discussed further below.

Disruption to the sarcomere has been described as a loss of myofibrillar integrity, specifically, Z-disk damage and A-band disorganisation (Friden and Lieber, 1998). Damage to the Z-disk has been evidenced in the elbow flexors following ECC exercise, with the non-exercised arm displaying significantly fewer disrupted or destroyed Z-disks (Lauritzen et al., 2009). Hansen et al. (2009b) produced similar findings following 100 maximal ECC actions of the knee extensors. The authors reported a significant increase in disrupted Z-lines at 5 and 24 h post-exercise, with a significant number of destroyed Z-lines at 24 h post-exercise. Friden et al. (1983) also suggested that maximal Z-line disruption was largest immediately post-exercise, with every third fibre disrupted, compared to 144 h post-exercise, with every tenth fibre disrupted. The initial damage to the Z-disk might involve Z-line streaming (Friden and Lieber, 1998) with the progression of the Z-band extending into the A-band, most likely due to a loss of Z-disk material, such as desmin and titin (Friden & Lieber, 1996, Friden, Sjostrom & Ekblom, 1983), with both structural proteins contributing to the force producing capacity of the muscle. Desmin loss following ECC exercise has been shown to occur within 5-15 minutes (Friden and Lieber, 1996) and at 1-3 days post exercise (Friden and Lieber, 1998), with titin shown to be significantly reduced at 24 h, following ECC exercise (Trappe et al., 2002). The loss of these structural proteins

could explain the loss of force producing capacity, commonly observed following ECC exercise (see section 2-5.2.1).

In addition to the ultrastructural damage to the sarcomere, muscle biopsy techniques can detect structural changes to the elements involved in the E-C process, namely, changes in t-tubule (Takekura et al., 2001, Yeung et al., 2002), and sarcoplasmic reticulum structure (Friden and Lieber, 1996), in the 2-3 days post ECC exercise, contributing to the disruption of the post-exercise disturbance in E-C coupling highlighted in section 2-4.2.1.

Although considered a gold standard for measuring markers of EIMD (Warren et al., 1999), biopsies also have limitations. For example, the procedure is uncomfortable for the participant, requires specialised storage and analysis of tissues and can exacerbate muscle damage through the sampling technique (Warren et al., 1999). Moreover, there are inherent issues with using such a small sample to measure damage for a whole muscle (Clarkson and Hubal, 2002). These limitations have resulted in researchers more commonly using indirect measures to quantify EIMD.

2-5.2 Indirect markers of Exercise Induced Muscle Damage

2-5.2.1 Loss of muscle function

A decline in the maximal amount of force a muscle can produce is one of the most commonly used indirect measures of EIMD and as of 1999, muscle force was reported in 50% of muscle damage studies reviewed by Warren et al. (1999). This is frequently in the form of a maximal voluntary contraction (MVC) which can either be isometric, concentric or eccentric. In a recovery context, this is an applicable method of assessing

EIMD, as athletes often implement strategies to resume maximal performance as quick as possible. Maximal isometric voluntary contractions (iMVC) are used routinely in muscle damage research as they are relatively easy to implement and have been shown to be a valid and reliable marker of EIMD (Warren et al., 1999, Damas et al., 2016). In addition, iMVCs have been shown to decrease immediately and in the days following EIMD in a range of muscle groups (Warren et al., 1999, Proske and Morgan, 2001, Chen et al., 2019). Reductions in force of 10-70% are commonly reported in the literature (Paulsen et al., 2010, Brown et al., 1997) with a return to baseline often not being achieved after seven days (Paulsen et al., 2012, Lavender and Nosaka, 2008, Goodall et al., 2017, Foure et al., 2019). The large range of force loss reported between studies can be partially explained by the responses varying between muscle groups examined. For example, the upper limb muscles produce greater losses in strength following ECC exercise than the lower limb muscles (Chen et al., 2019). Additionally, the loss in strength following damaging exercise is affected by both the nature of the damaging stimulus (Sayers et al., 2003). For example, high-force, high volume ECC muscle actions of isolated muscle groups produce the greatest and most lengthy reductions in iMVC compared to exercise involving a high metabolic demand such as marathon running (Paulsen et al., 2012). However, variations do exist within (Nosaka and Clarkson, 1996) and between studies (Vincent and Vincent, 1997, Clarkson and Hubal, 2002), although these variations are more attributable to the response to the muscle damaging stimulus (Baumert et al., 2016), not due to the variability of iMVC as a measure, which has been shown to be reliable (Warren et al., 1999).

The explanation for loss of force following ECC exercise has been attributable to several theories, including, but not limited to, peripheral and central mechanisms.

Central mechanisms that cause a reduction in force producing capacity can occur from a reduction in neural drive to the muscle from the brain, including a reduction in motor neuron firing rate (Sayers et al., 2003, Hubal et al., 2007). Recently, SOURON (2018) reported a significant reduction in voluntary activation (VA) and iMVC in the knee extensors, measured via the interpolated twitch technique, immediately following a bout of ECC exercise. However, VA had returned to pre-exercise levels by 1 h post-exercise, indicating that central fatigue is present in the time immediately following exercise. Souron et al. (2018) also reported iMVC still being significantly reduced at 96 h post-exercise, despite VA being at pre-exercise levels, with the authors claiming that peripheral, not central mechanism are responsible for the loss in force 1 h + after ECC exercise. Conversely, Goodall et al. (2017), reported that VA relative to pre-exercise levels did not recover until 48 h post-ECC exercise and iMVC force loss persisted after seven days post-exercise, indicating that central mechanisms played a part in force decrement until at least 24 h after exercise. The study by Souron et al. (2018) examined the quadriceps whilst Goodall et al. (2017) examined the biceps, which is the notable difference between the studies. It is possible that the higher susceptibility of the upper limbs to EIMD than the lower limbs (Chen et al., 2019) is the reason for the difference in VA recovery between the studies. Nevertheless, the iMVC force loss in the studies by Souron et al. (2018) and Goodall et al. (2017) persisted past the point of VA recovery, suggesting that peripheral damage is responsible for prolonged force decrements following ECC exercise.

Peripheral mechanisms, which have previously discussed in this chapter, include structural damage to the contractile elements of the muscle, or a disruption of the E-C coupling process, which Warren et al. (1999) suggested could be responsible for an

immediate loss of muscle force following damaging exercise, due to a reduced amount of CA^{2+} released per action potential (Balnave and Allen, 1996, Warren et al., 1993b). However, these findings were from mouse muscle fibres, which were not replicable in amphibian muscle (Morgan et al., 1996). In human studies, peripheral mechanisms of force loss can be measured indirectly by the use of the potentiated twitch technique, whereby an electrical impulse is delivered to the muscle or nerve, following a maximal contraction, with the forces subsequently recorded (Oskouei et al., 2003). This method of measuring the function of the contractile elements of the muscle has been shown to be reliable in the biceps (Todd et al., 2004) and quadriceps (Morton et al., 2005). Using this technique, Goodall et al. (2017) found that potentiated twitch was reduced at seven days post-ECC exercise, despite VA recovering by 48 h, giving further indication that peripheral and not central mechanisms are responsible for the prolonged force loss after a damaging stimulus. The evidence presented suggests that there is a mixture of central and peripheral mechanisms responsible for the force loss suffered after ECC exercise, with the central element unlikely to contribute past 48 h post-exercise. However, the exact contributions of the two mechanisms remain unclear and warrant further investigation.

2-5.2.2 Intramuscular proteins: Creatine kinase

Circulating concentrations of specific intramuscular proteins, such as CK, myoglobin, lactate dehydrogenase and troponin, are commonly measured as an indirect marker of EIMD (Clarkson and Hubal, 2002, Brancaccio et al., 2010). The measure of CK is the most common in EIMD literature (Baird et al., 2012) and is the only intramuscular

protein measured in this thesis, therefore, the other markers mentioned will not be discussed; for an overview the reader is directed to Warren et al. (1999).

Creatine kinase is a dimeric protein responsible for catalysing the reversible exchange of phosphocreatine and adenosine diphosphate (Brancaccio et al., 2010). There are at least five isoforms of CK, three of which are located in the cytoplasm; (1) CK-BB, found in the brain, (2) CK-MB, found in cardiac muscle and (3) CK-MM, which is specific to the muscle (Dawson and Fine, 1967, Baird et al., 2012). For the remainder of this thesis, CK will refer to the muscle specific isoform.

Creatine kinase is present in a number of domains of the myofiber where ATP consumption is high, and specifically is bound to the M-line structure of the sarcomere (Hornemann et al., 2003), meaning that exercise that damages the muscle fibre structure at the sarcolemma and Z-disk level produces a decrease in membrane permeability and an increase in total serum CK (Noakes, 1987, Epstein, 1995). Circulating levels of CK in healthy individuals are generally <175 $IU \cdot L^{-1}$. The degree to which serum levels of CK are raised after exercise is determined by several factors. For example, the highest immediate post-exercise CK levels are produced by long duration exercise with repetitive impact forces, such as marathon running (Nuviala et al., 1992) or triathlon events (Denvir et al., 1999). However, the absolute levels of CK following this type of exercise are relatively low (100-1000 $IU \cdot L^{-1}$) and peak at ~24 hours post exercise (Sorichter et al., 2001, Malm et al., 2004). High-intensity exercise with a large ECC component produces the largest and most prolonged rise in serum CK levels (Baird et al., 2012), especially isolated muscle groups of the upper body (Hyldahl et al., 2011), producing serum CK levels of 4000-6000 $IU \cdot L^{-1}$ at 96 hours

post-exercise (Hirose et al., 2004). With such a large detectable response, it is unsurprising that CK is a commonly used indirect marker of EIMD. It should be noted however, that even in participant groups with closely matched characteristics, CK is subject to high inter-subject variability (Paulsen et al., 2012). Additionally, increases in serum CK do not always correlate with other markers of EIMD (Warren et al., 1999, Damas et al., 2016), such as Z-line disruption (Stupka et al., 2000) and loss of muscle function (Fridén and Lieber, 2001). Although a commonly utilised and useful measure, the limitations of CK measurement following EIMD must be considered and not used to quantify the magnitude of EIMD. Ideally, CK measures should be used in conjunction with other direct or indirect markers, to further understand the magnitude of the EIMD.

2-5.2.3 Muscle soreness

Exercise that involves unaccustomed movements with a large ECC component typically produce muscle soreness (Asmussen, 1956), which is typically measured as a marker of EIMD (Warren et al., 1999). The time-course of muscle soreness varies between individuals; however, soreness typically appears between 12-24 h post exercise, peaks between 24-72 h and is alleviated after seven days but can last longer (Newham, 1988, Graven-Nielsen and Arendt-Nielsen, 2003, Armstrong, 1984). Due to the non-immediate manifestation of the sensation, the term delayed onset of muscle soreness (DOMS) is used.

The mechanism responsible for the appearance of DOMS remains unclear, however several theories attempt to explain this. One theory is that the build of lactic acid after the cessation of exercise causes an excess of metabolic ‘waste product’, leading to a

noxious stimulus and the sensation of pain (MacIntyre et al., 1995, Gulick et al., 1996, Armstrong, 1984). However, the lactic acid theory has been strongly discredited, due to CON exercise producing high levels of lactic acid with no accompanying DOMS and ECC exercise producing high levels of DOMS with little/no change in lactic acid (Schwane et al., 1983). Other mechanisms proposed to be the cause of DOMS, albeit derived from animal studies, include an increase in resting muscle electromyography (EMG) activity (De Vries, 1966), damage to the connective tissue (Lieber and Friden, 1988), or the involvement of neurotrophic factors, such as the post-exercise activation of the B2 bradykinin receptor-nerve growth factor and cyclooxygenase-2 pathways (Murase et al., 2013).

The measurement of DOMS is often conducted via the use of a visual analogue scale (VAS) (MacIntyre et al., 1995), which requires participants to rate their perception of soreness on a fixed scale, typically, 0 mm (no pain at all) to 100 mm (unbearable pain) (Chen et al., 2016). These perceived ratings of soreness can be performed passively (seated or supine), actively (during contraction) or during the application of a set pressure (Lau et al., 2015). A common criticism of the VAS technique is that participants might be prone to biasing their score, based on previous measurements, particularly if measurements are taken on consecutive days (MacIntyre et al., 1995). Additionally, it is important to note that the time course and intensity of DOMS does not always correlate with other direct or indirect markers of EIMD, such as muscle function (Nosaka et al., 2002) and serum CK levels (Hyldahl and Hubal, 2014). Nevertheless, measuring DOMS is a useful practice, due to the influence of perceived soreness on subsequent athletic performance (Fletcher et al., 2016), however this

should not be a stand-alone measure and should be used in conjunction with other markers of EIMD (Paulsen et al., 2012).

There is a multitude of ways to measure the EIMD response to exercise, all of which have applications and limitations. Basing the EIMD response on one particular measure would not be appropriate, given the lack of correlation between various markers of EIMD and the variation between studies (Warren et al., 1999). Therefore, it is advisable that a battery of tests are performed within EIMD research so that more valid conclusions can be drawn from the data and comparisons between the literature can be performed.

2-6 DETERMINANTS OF EXERCISE INDUCED MUSCLE DAMAGE

As previously mentioned, variations in the response to a damaging bout of ECC exercise exist within (Nosaka and Clarkson, 1996) and between studies (Vincent et al., 2010, Clarkson et al., 2005), that are not attributable to methodological inconsistencies. Furthermore, participants have previously been categorised into high and low responders to the same bout of exercise, after measuring force loss (Hubal et al., 2007) and the CK response (Isaacs et al., 2019, Brancaccio et al., 2007). Nevertheless, there is strong evidence to suggest that differences in the damage inducing stimulus can produce varying responses in markers of EIMD. The following section will discuss these determinants.

2-6.1.1 High fascicle strain

The ‘popping sarcomere’ theory, where muscle fibres are stretched under forceful actions on the descending limb of the LT curve (Morgan, 1990), might be an explanation as to why EIMD occurs following ECC exercise. This theory is supported by the notion that excessive fascicle strain, defined as the amount of lengthening beyond the optimal fascicle length for maximal isometric force production (Lieber and Friden, 1993), is a determinant of EIMD. For example, Lieber and Friden (1993) reported that isolated rabbit tibialis anterior muscle fibres subjected to 25% strain produced significantly lower levels of tetanic force post exercise, compared to fibres at 12.5% strain. Data from Talbot and Morgan (1998) supported the conclusions from Lieber and Friden (1993), finding that a greater amplitude of strain resulted in a greater fall in post-contraction active tension in isolated toad fibres. Contrary to these findings, Warren et al., (1993a) concluded that during five ECC contractions at 110, 120 and 130% of muscle length at peak isometric tetanic tension, the theory that initial length of the muscle fibre elicited a greater loss in sarcolemma integrity, as well as increased markers of EIMD (CK), was not supported.. One explanation for the difference in findings could be that Warren et al., (1993a) only used five eccentric contractions to elicit EIMD, in comparison to 900 in the study by Lieber and Friden (1993). In addition, both Lieber and Friden (1993) and Talbot and Morgan (1998) measured the optimal length to maximise force production, allowing a quantification of the amount of strain applied to the fibre. Warren et al., (1993a) did not measure this, therefore it is difficult to ascertain the proportion of muscle lengthening that is on the descending limb of the LT curve, a key factor in the popping sarcomere theory (Morgan, 1990) The studies by Lieber and Friden (1993), Talbot and Morgan (1998) and Warren et al., (1993a) used *in vitro* experimental conditions on rabbit, toad and

rat muscle fibres, respectively, to draw their conclusions, which makes it difficult to extrapolate to a human muscle *in vivo*.

Translating fascicle strain and EIMD data from *in vitro* and animal studies is not straight-forward due to key methodological differences. Studies using these methods typically only use a small number of fibres which are stretched beyond their normal physiological range and under a non-voluntary, electro-induced stretch (Butterfield, 2010, Brooks et al., 1995), something that is not reflective of muscle and tendon unit behaviour *in vivo*.

For studies using *in vivo* voluntary activation of muscles, the elastic behaviour of the MTU creates a more complicated EIMD process which cannot be explained by data from single fibre studies (Butterfield, 2010). *In vivo* studies that have measured fascicle behaviour during ECC exercise and the magnitude of EIMD have produced varying results. For example, following 300 maximal ECC contractions of the plantar flexors, Guilhem et al. (2016) found a significant correlation ($r = -0.47$, $P < 0.05$) between maximal fascicle lengthening (0.55 ± 0.16 cm) and the decrease in force loss (~15%) at 48 h post-exercise. In a study investigating the amount of fascicle lengthening and EIMD in the VL following 72 maximal ECC contractions, Hicks et al. (2017) reported a significant correlation between the relative change in VL fascicle length ($59.4 \pm 12.0\%$) and the relative change in CK ($883 \pm 667\%$) from pre to peak levels. However, the authors found no significant correlations between the magnitude of iMVC torque loss and the amount of fascicle lengthening at any time-point, albeit a significant reduction in iMVC force was reported between pre-damage (264 ± 35 N·m) and 48 h post-exercise (221.0 ± 48.4 N·m, $P = 0.004$) (Hicks et al., 2017).

Conversely, Penailillo et al. (2015), reported that fascicles in the VL lengthened by 8.0 cm on average during sub-maximal, ECC cycling exercise, resulting in no loss of iMVC torque at 48 h post-exercise. Interestingly, Hoffman et al. (2014) found that one hour of backward downhill walking induced a ~23% loss in iMVC. However, this loss in force could not be explained by the amount of fascicle lengthening of the medial gastrocnemius as there was only a relatively small stretch amplitude (~18% of optimal fascicle length), occurring predominantly on the ascending limb and plateau regions of the LT curve.

The inconsistencies in the relationship between fascicle lengthening and markers of EIMD make drawing firm conclusions challenging, however, the differences in study design might explain the discrepancies. For example, in the two studies involving the ankle joint, Guilhem et al. (2016) reported a fascicle lengthening of 1.36 × fascicle slack length [the muscle length beyond which the muscle begins to develop passive elastic force (Guilhem et al., (2016)] during maximal ECC exercise, whereas Hoffman et al. (2014) reported fascicle lengthening of 1.18 optimal length during the downhill backward walking protocol. This additional stretch could account for the lack of iMVC force reduction seen by Hoffman et al. (2014). It must be noted however, that both studies normalised fascicle lengths differently, therefore direct comparisons of fascicle lengthening can be problematic, especially as particularly short fascicle slack lengths occur in some participants (Guilhem et al., 2016). Despite the greater amount of relative fascicle lengthening in the study by Hicks et al. (2017) in comparison to Guilhem et al. (2016) (79% vs 51%, respectively), Hicks did not report a significant correlation between fascicle lengthening and iMVC force loss, whereas Guilhem et al. (2016) did. Most likely, the end range of motion of 90° knee angle in the study by

Hicks et al. (2017) was well within the normal physiological range of the knee joint. Guilhem et al. (2016) employed a range of motion that was closer to the end physiological range of the ankle joint, although the differences in the range of motion used by the two studies might have been mitigated by differences in the lengthening of the patella and Achilles tendons. Nevertheless, previous research typically shows that ECC exercise at long muscle lengths induces a greater EIMD response (Nosaka and Sakamoto, 2001, Child et al., 1998). However, this is not always the case as Penailillo et al. (2015) performed exercise at a knee angle cycling from 20° to 120° knee angle, 30° more than the range of motion employed by Hicks et al. (2017). Although no correlations between fascicle lengthening and post-exercise iMVC force loss was reported in either study, Hicks et al. (2017) still reported a significant loss in iMVC force from baseline, post ECC whereas Penailillo et al. (2015), reported no such loss in force. The most likely explanation is that the submaximal nature of the ECC cycling protocol in the study by Penailillo et al. (2015), resulted in lower resistance of fascicles to active stretch, due to lower muscle activity levels, resulting in high fascicle lengthening in lieu of a force impairment post-exercise.

The varying response to fascicle lengthening and markers of EIMD, specifically the loss of iMVC force, seem to be determined by a number of factors including, the joint studied (knee vs ankle), the proximity to end physiological range (i.e. maximal fascicle lengthening) and the intensity of the exercise protocol (maximal vs submaximal), which will be discussed in the next section.

2-6.1.2 High torque

Eccentric muscle actions can lead to EIMD, especially if an unaccustomed movement is performed (Proske and Morgan, 2001). What is less clear is how differing intensities of ECC muscle action produce differing magnitudes of EIMD. Nosaka and Newton (2002) was the first to investigate *in vivo* the effect of maximal and submaximal ECC on markers of EIMD in the elbow flexors. Participants performed three sets of 10 repetitions at either maximal effort or at 50% of iMVC. It was reported that at 1-day post-exercise, iMVC force recovery was significantly higher in the 50% group (80.8 ± 5.3%) vs the maximal group (54.1 ± 9.7%). This significant difference in iMVC recovery was still present at day five post-exercise, with the 50% group recovering to a greater extent than the maximal group (92.7 ± 5.5% vs 68.7 ± 6.3%, respectively). Paschalis et al. (2005) adopted a similar approach to test the effect of high (maximal) and low (50% of maximum) ECC contractions on EIMD in the knee extensors. Participants performed 12 sets of 10 maximal ECC actions in one bout, followed by continuous ECC exercise at 50% of maximum ECC torque on the contralateral leg two weeks later. The sub-maximal exercise protocol was continued until the total work was approximately equal to the maximal session. Paschalis et al. (2005) reported that the muscle damage response, measured by iMVC, between the maximal and sub-maximal exercises was different at each time-point post-exercise. However, the only significant reduction of force producing capacity was recorded at 24 hours post-exercise in the maximal intensity group. It must be noted that the muscle following the continuous ECC bout might have suffered less damage due to the contralateral RBE (Howatson and van Someren, 2007) (see section 2-7), therefore the results of this study should be interpreted with some caution. Barroso et al. (2011) investigated the

effect of intensity on markers of EIMD by asking three groups of participants to perform one of the following protocols on the elbow flexors: 1) 5 sets of 6 ECC actions at 70% 1 repetition max (1RM), 2) 10 sets of 6 ECC actions at 70% 1RM, 3) 5 sets of 6 ECC actions at 110% of 1RM. It was reported that force producing capacity had returned to pre-exercise levels after 48 h for all groups but only the group exercising at 110% 1RM showed significantly higher DOMS and lower range of motion at 48 hours post exercise, in comparison to baseline. In contrast, Peake et al. (2006) reported that submaximal ECC exercise (10 sets of 60 ECC actions at 10% peak iMVC) resulted in a significantly lower loss of force at 24, 48, 72 and 96 hours, compared to maximal ECC exercise with a lower volume (10 sets of three maximal ECC actions). Additionally, isometric strength in the submaximal group had returned to pre-exercise levels by 48 hours, whereas strength in the maximal ECC group had still not returned to pre-exercise levels by 96 hours, indicating a prolonged effect of higher ECC torque and lower volume on loss of muscle function.

Evidence from previous studies fails to give a conclusive answer as to whether higher levels of ECC torque produce greater magnitudes of EIMD. Paschalis et al. (2005) argued that the reduced difference between high and low intensity exercise and markers of EIMD, in comparison to Nosaka and Newton (2002) could be due to participants in the latter study not performing similar amounts of total work. However, Gauche et al. (2009) had participants perform work matched bouts of high (80% max ECC torque) or low (40% max ECC torque) and found no differences in markers of EIMD between groups. Another argument against total work being the main driver of increased markers of EIMD is that Barroso et al. (2011) found no difference between high or low intensity groups, despite the high intensity group performing more total

work. In addition, participants in the low intensity group in the study by Peake et al. (2006) performed 6791 ± 187 J vs 1288 ± 36 J in the high intensity group. Despite the higher amount of total work done in the low intensity group, the force loss was significantly lower than the high intensity group at all time points. However, Peake et al. (2006) implemented a submaximal intensity of 10% peak iMVC, creating a difference between groups of 90% intensity, whereas other studies have used differences in intensities of 40% (Gauche et al., 2009, Barroso et al., 2011) and 50% (Paschalis et al., 2005, Nosaka and Newton, 2002), indicating that a greater difference in intensity, irrespective of total work done, produces greater differences in the magnitude of EIMD recorded. Whilst no studies have reported that lower ECC torques produced greater magnitudes of EIMD than high ECC torque, further study is required to identify the specific effect of ECC torque on EIMD markers.

2-6.2 Tendon as a Determinant of exercise induced muscle damage

2-6.2.1 – Tendon properties and measurement technique

The structure of the human tendon is dominated by collagen molecules that are organized in a strict hierarchical manner, that results in the formation of collagen fibrils, which group together to form tendon fascicle bundles, separated by an endotenon (Kastelic et al., 1978). Numerous fascicle bundles then group together to form the tendon unit, which is surrounded by the epitenon (Figure 2-5) (Wang, 2006). Collagen molecules are approximately 1.5 nm in diameter and 300 nm in length and are organised into a precise pattern, stabilised by covalent intramolecular cross-links that bind the collagen molecules together (Kadler et al., 1996, Bailey, 2001). Type I collagen is the predominant collagen protein in tendon structure (Riley, 2005),

accounting for 60% of tendon dry mass and 95% of total collagen (Riley et al., 1994, Evans and Barbenel, 1975). Type III and Type V collagens make up the remaining 5% of tendon collagen, with type III collagen predominantly found in the epitenon and endotenon (Duance et al., 1977). Tendon collagen is configured in a unique pattern that appears wavy under light microscope (Figure 2-6a), appearing as a "crimp pattern" (Whittaker and Canham, 1991, Stouffer et al., 1985), which is lost upon stretching of the tendon (Figure 2-6b) but returns if <4% stretch of the tendon is performed (Rigby et al., 1959). This recovery of the crimped region of the tendon might be due to the elastic fibres (Butler et al., 1978) which are made up of microfibrillar proteins, and elastin, with the latter making up ~2% of the tendon dry weight (Jozsa et al., 1989). Stretches of beyond 4% can result in microscopic tearing of the tendon, whereas stretch beyond 8-10% can lead to macroscopic failure (Butler et al., 1978), however there is reason to believe that these percentages might be underestimated, due to *in vitro* evidence suggesting that 14% stretch can be applied to avian wing tendons (Devkota and Weinhold, 2003) and human patella tendons, without rupture (Johnson et al., 1994). However, much lower strain percentages of between 5-6% have been reported in the human patella tendon *in vivo*, with this low strain unlikely to be at a point where the tendon is approaching failure during measurement (Hansen et al., 2006, Carroll et al., 2008). The amount of stretch applied to the tendon determines stiffness, with tendon stiffness being the property associated with the ability for the tendon to transmit force effectively (Onambele et al., 2007). Tendon stiffness is calculated by the amount of force applied to the tendon divided by the change in tendon length from starting length, however, these stiffness measures do not account for the anatomical properties of the tendon such as cross-sectional area

(CSA). To account for this, Young's Modulus (YM) can be calculated by multiplying the tendon stiffness value by the ratio of tendon length to tendon CSA (Maganaris and Paul, 1999).

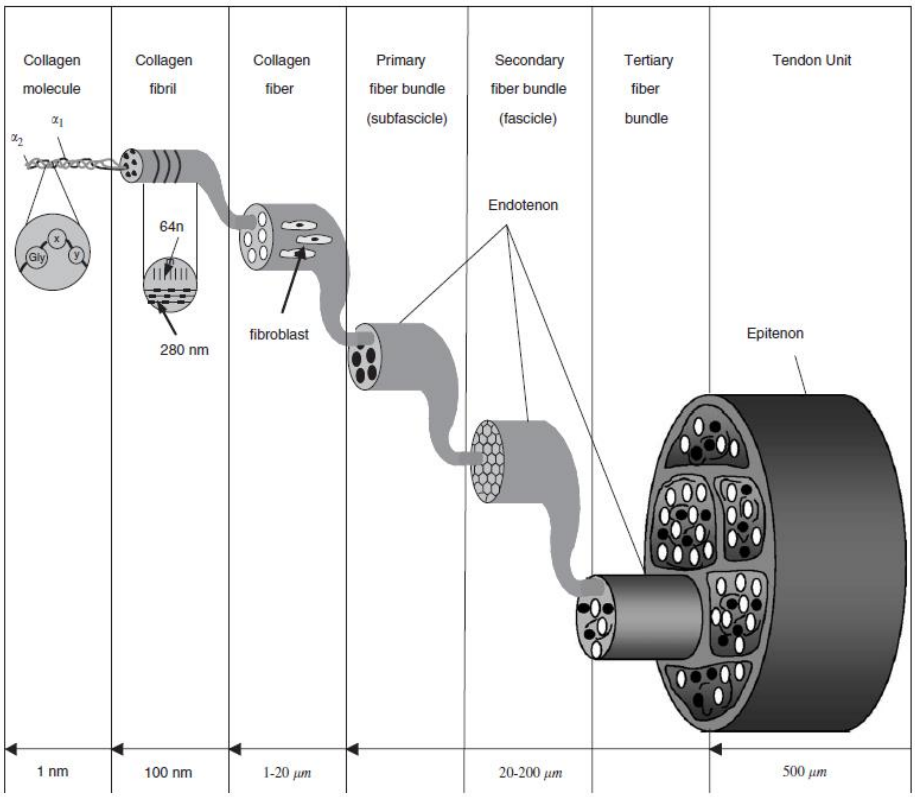

Figure 2-5 Tendon structure. Modified from Silver et al. (2003).

Modern methods of measuring *in vivo* mechanical properties of tendon are predominantly performed using B-mode ultrasound, during graded isometric contractions (Fukashiro et al., 1995). When essential precautions are taken, ultrasound-based measures of tendon properties are reliable and able to detect changes in the mechanical properties of tendons (Hansen et al., 2006, Schulze et al., 2012). Despite this, studies with similar methodologies can produce varying differences in material properties of tendon, for example a 30% variation in patella tendon properties (Couppe et al., 2009, Seynnes et al., 2015), highlighting the importance of consistency

between trials. Several factors could influence the reliability of ultrasound-based tendon measurements such as 1) Tracking, where the ultrasound probe is misaligned longitudinally, producing an overestimation of tendon elongation (Magnusson et al., 2001), 2) Scanning, or more specifically, incomplete scanning; for example, if tibial movement is not accounted for in the measurement of patella tendon elongation, strain can be underestimated by 38-45%, meaning that both the proximal and distal end of the patella tendon needs to be measured (Onambele et al., 2007, Hansen et al., 2006), 3) Tracking technique, which mainly includes slight movements of the US transducer during contraction, which can influence the grey-scale pattern of the images produced, subsequently influencing data analysis (Seynnes et al., 2015) and 4) Estimation of tendon force, specifically due to estimations of patella tendon moment arm (PTMA) which can see variations of 40-50% between different methods (Tsaopoulos et al., 2006) which could lead to errors of up to 67% in calculated tendon force (Seynnes et al., 2015). As previously mentioned, the use of ultrasound to measure tendon properties is reliable (Stenroth et al., 2019, Gellhorn and Carlson, 2013), however there must be a high level of precaution taken to ensure consistent technique, as well as an understanding that results might not be transferable to other studies due to methodological differences (Seynnes et al., 2015). Nevertheless, the use of ultrasound as an imaging technique has allowed research into the interaction of the muscle and tendon during contraction and specifically, how this might affect the magnitude of EIMD experience during ECC exercise.

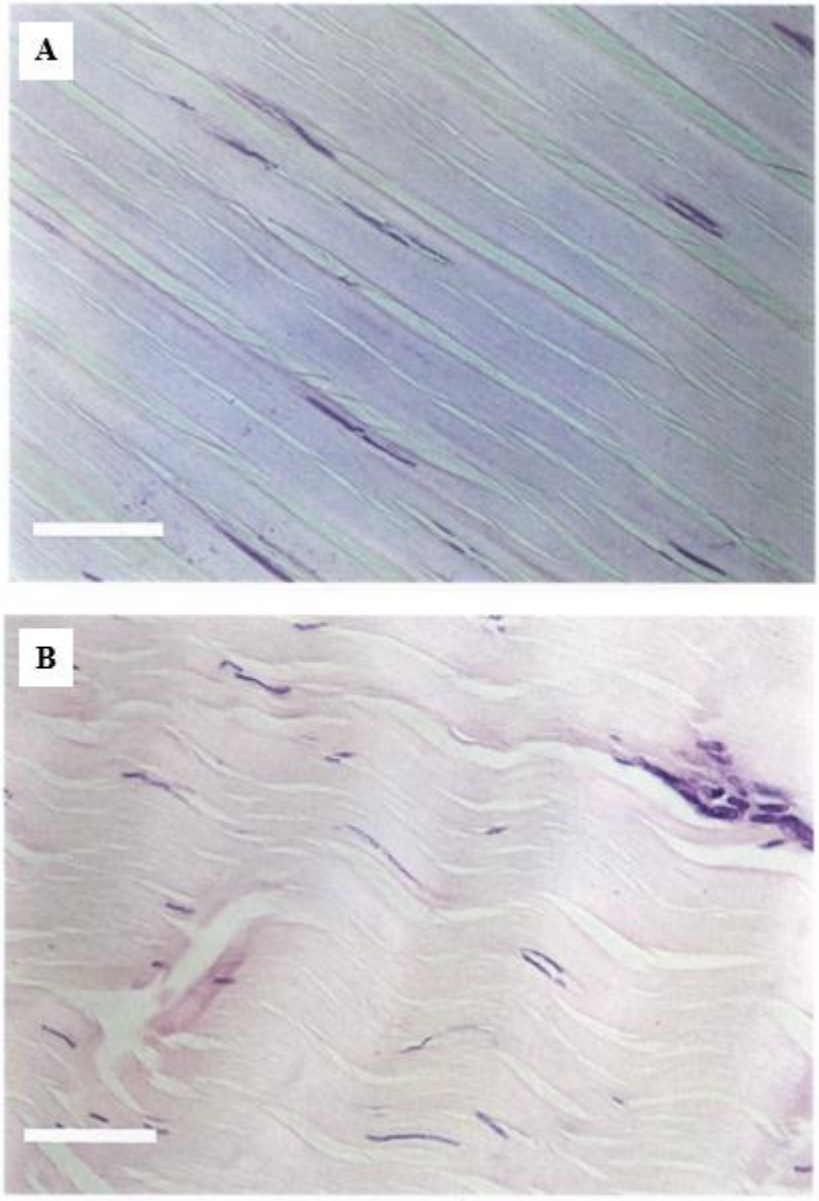

Figure 2-6 Strained (A) and unstrained (B) Achilles tendon, using bright fields of view stained with hematoxylin and eosin. Adapted from Whittaker and Canham, (1991).

2-7.2.2 The role of the tendon in force transmission and dissipation

Research has suggested that the elastic properties of the tendon serve as a means of conserving energy during locomotion (Roberts, 2002, Cavagna, 1977) and increasing the power output of a muscle when required (Bobbert et al., 1986, Alexander, 2002). For humans, the differing roles that a tendon might play in specific situations will

require differences in stiffness, depending on the action (e.g. running vs maximal strength exercises). These differences in tendon stiffness are evident across various muscles in the body (Ker et al., 1988) and is highly dependent on muscle and tendon architecture (Roberts, 2002). For example, muscles that are involved with running and walking (i.e. the *Soleus* and *Gastrocnemius*) tend to have short fibres with high pennation angles and a long tendon (i.e. the Achilles tendon). During this kind of action, muscle fascicles produce less work by remaining quasi-isometrically contracted (or shortening) despite total MTU lengthening (Fukunaga et al., 2002), indicating that the tendon acts to optimise the efficiency of the muscle. For example, Reeves and Narici (2003) reported that muscle fascicles in the *tibialis anterior* contracted quasi-isometrically during ECC contractions in an isokinetic dynamometer, independently of angular velocity. The authors attributed this behaviour to the elongation of the tendon, outlining the implications in energy saving, releasing stored energy during tendon recoil following ground contact. Additionally, Spanjaard et al. (2007) found that during stair descent, most of the muscle fascicle lengthening occurred in the late phase of the step, when little MTU changes occurred. Once again, indicating that there is a significant influence of the tendon in energy absorption (Roberts and Konow, 2013).

Although it is evident that the tendon acts to increase the metabolic efficiency of the muscle, it is important to note that this behaviour can vary across movement types and intensities. For example, Kouno et al. (2019) found that in both the plantar flexors and the knee extensors, the behaviour observed in the tendon was different between ramped contractions and ballistic exercise. Specifically, Kouno et al. (2019) reported that hysteresis was lower and tendon elongation was higher in ramped vs ballistic

exercise. This therefore has implications for the extrapolation of tendon behaviour from ramped contractions to ballistic movements, creating the need to revalidate tendon properties during other types of movement (Kouno et al., 2019). Moreover, the behaviour of the MTU can be altered despite the same exercise being performed, which was recently demonstrated by Hollville et al. (2019). The authors investigated the differences in MTU behaviour of the *gastrocnemius medialis* (GM) and VL during double-leg and single-leg landing from two differing heights using US. It was found that for both the GM and VL, single-leg landings induced a greater amount of tendon stretch than double-leg landings, protecting fascicles from high ECC stress. Interestingly, the effect of drop height (25 cm, 50 cm and 75 cm) produced no difference in GM fascicle lengthening amplitude, whereas VL fascicles increased their contribution to total MTU lengthening as drop height increased. This indicates that the longer, more compliant Achilles tendon (AT) protected the GM fascicles from increased lengthening, despite the increased height, whereas the patella tendon did not protect VL fascicles in the same manner. These findings by Hollville et al. (2019) indicated the need to understand how exercise intensity can alter MTU behaviour differently across different muscle groups. Something that must be taken into consideration when interpreting results from studies with different exercise modalities.

In addition to the tendon acting as an energy efficiency vehicle, research has suggested that the tendon acts as a mechanical buffer during ECC exercise in the lower leg, providing a protective mechanism against markers of EIMD (Roberts and Azizi, 2010, Roberts and Konow, 2013). During backwards downhill walking, Hoffman et al. (2014), found that ~91% of MTU lengthening was attributed to stretch in the AT, which agrees with findings in similar studies in animals (Azizi and Roberts, 2010).

Hoffman et al. (2014) suggested that this high degree of AT contribution to total MTU lengthening might reduce the amount of EIMD experienced, however, markers of EIMD over two h post-exercise were not measured; therefore, the longer-term implications of this study require investigation. More recently, this mechanical buffering behaviour of the AT was suggested to reduce the magnitude of EIMD experienced during 30 maximal ECC actions on an isokinetic dynamometer (Guilhem et al., 2016). Most notably, the authors reported a significant correlation ($r = 0.68$, $P < 0.05$), between negative muscle fascicle work [i.e. fascicle braking work; (Azizi & Roberts 2014)] beyond slack length and the amount of iMVC force decrement at 48 hours post exercise. Additionally, the authors stated that AT compliance reduced the amount of strain on the fascicle, attenuating the amount of EIMD experienced (Guilhem et al., 2016). This relationship between tendon behaviour and the reduction in magnitude of EIMD experienced was also demonstrated in the upper leg by both McHugh et al. (1999b) and Marginson et al. (2005), who both attributed lower magnitudes of EIMD in groups with more compliant MTU's in the hamstrings and quadriceps, respectively. This pattern was repeated in the elbow flexors in a study by Chen et al. (2014), when the authors reported a lower magnitude of EIMD experienced by pre-adolescent males, in comparison to adolescent and adult males, following 30 maximal ECC actions on an isokinetic dynamometer. Chen et al. (2014) suggested that higher muscle mass and greater mechanical strain in the adolescent and adult groups vs the pre-adolescent group was unlikely to explain the difference in markers of EIMD. However, the authors suggested that, in line with Marginson et al. (2005), the lower magnitude of EIMD experienced by the pre-adolescent group was due to differences in the MTU. Although differences in EIMD magnitude between groups

might be explained by MTU behaviour, it must be noted that McHugh et al. (1999b), Marginson et al. (2005) and Chen et al. (2014) did not directly measure tendon stiffness and conclusions drawn from these studies must therefore be interpreted with some caution.

As previously mentioned, there is evidence to suggest that MTU behaviour can affect markers of EIMD following damaging exercise, albeit from indirect interpretations (McHugh et al., 1999b, Marginson et al., 2005, Chen et al., 2014). Recent research by Hicks et al. (2013) has investigated this relationship directly. During 12 maximal ECC actions of the knee extensors, Hicks et al. (2013) reported that increased muscle fascicle lengthening in the VL during maximal ECC exercise was significantly correlated with higher patella tendon stiffness ($r = 0.476$, $P = 0.023$) and YM ($r = 0.470$, $P = 0.049$). Although Hicks et al. (2013) did not measure markers of EIMD, the same authors identified a significant correlation between VL fascicle lengthening and the subsequent CK response (Hicks et al., 2017), following 6 sets of 12 maximal ECC actions of the knee extensors. Although the authors in this study found no relationship between MTU properties and a loss in muscle function, there was a correlation trend between estimated patella tendon lengthening during ECC exercise and peak CK response ($r = -0.41$, $P = 0.06$). This trend between increased patella tendon lengthening and reduced markers of EIMD, taken together with a greater CK response with increased VL fascicle lengthening provides some evidence that the tendon can mediate the muscle damage response following maximal ECC exercise of the knee extensors, albeit limited to CK response.

From the evidence provided, there is a clear indication that differences in MTU behaviour that are directly measured within groups (Hicks et al., 2017, Guilhem et al., 2016) or indirectly assumed between groups (McHugh et al., 1999b, Marginson et al., 2005, Chen et al., 2014) have an effect on the magnitude of EIMD experienced following ECC exercise.

Therefore, if there is potential for MTU behaviour to be altered by a specific exercise modality, such as maximal ECC exercise (Penailillo et al., 2015, Lau et al., 2015), further investigation is warranted to understand the relationship between MTU mechanics and subsequent exercise response.

2-7.3 Oestrogen as a determinant of exercise induced muscle damage

2-7.3.1 The structure and function of oestrogen in eumenorrheic females

Oestrogens are a group of steroid hormones predominantly found within females, which primary functions include the development and maintenance of the reproductive system, the cyclic production and release of the ovum, and the development of secondary sex characteristics (Sherwood, 2016). The actions of oestrogen are mediated by binding to specific receptor sites that are localised in tendons, ligaments, and skeletal muscle tissue (Hansen, 2018). These tissue receptors include specific nuclear oestrogen receptors (OR) α and β, and plasma-membrane OR α and β which act as transcription factors once bound to oestrogen (Wiik et al., 2003, Wiik et al., 2005, Sciore et al., 1998, Hart et al., 1998, Faryniarz et al., 2006). This expression of OR within muscle and tendon suggests that oestrogen can have a direct effect on these tissues.

Oestrogens as a term refers to three hormones that are structurally similar, these are estradiol-17β, estrone and estriol. Estradiol-17β has the highest estrogenic properties and is the most abundant in humans (Tepperman, 1987, Kendall and Eston, 2002). In females, most oestrogens are synthesised in the ovaries. Estradiol-17β is synthesised from testosterone and estrone form androstenedione (Thomas and Potter, 2013), with estriol being primarily synthesised in the placenta during pregnancy (Tepperman, 1987). Although predominantly a female hormone, oestrogens are still synthesised in small quantities in males via the testes and adrenal glands (Velle, 1966).

In menstruating females, oestrogen levels fluctuate throughout the menstrual cycle (typically lasting ~28 days) which occurs repeatedly from menarche, arising approximately at age 13 (Whincup et al., 2001) until post menopause, at approximately age 51 (Rosner and Colditz, 2011). The menstrual cycle is divided up into three main phases where the ratio of oestrogen and progesterone levels substantially differ: the follicular phase, the ovulation phase and the luteal phase (Maki et al., 2002), which can be further divided for specificity. During the early follicular phase, oestrogen and progesterone levels are low (~150 pmol·L^{-1} and 0.6 nmol·L^{-1}, respectively). During the late follicular phase, oestrogen and levels begin to rise (~450 pmol·L^{-1}) while progesterone levels remain low (~0.6 nmol·L^{-1}). At ovulation, oestrogen levels remain high (~671 pmol·L^{-1}) whilst progesterone levels begin to rise (~2.5 nmol·L^{-1}). During the luteal phase, oestrogen levels are approximately 313, 495 and 327 pmol·L^{-1} at the early, mid and late stages, respectively, while progesterone levels are approximately 14, 36 and 14 nmol·L^{-1} at the same respective stages in the luteal phase (Stricker et al., 2006). In addition to their role in reproductive function and health, oestrogens have unique properties that have been associated with attenuating the magnitude of EIMD

following strenuous exercise (Kendall and Eston, 2002), which will be discussed further below.

2-7.3.2 The antioxidant properties of oestrogen

Earlier in this chapter (section 2-4.2.3) it was discussed that RONS might play a role in exacerbating markers of EIMD following ECC exercise, most likely manifesting as secondary muscle damage (Zerba et al., 1990). It was suggested within the same section that an environment containing compounds with antioxidative properties might attenuate these heightened markers of EIMD, compounds such as oestrogen (Tiidus et al., 2005, Sugioka et al., 1987). The phenolic ring of oestrogen is similar to tocopherol, which displays strong antioxidant behaviour (Tiidus et al., 1999, Bar and Amelink, 1997). The structural similarities between oestrogen and tocopherol which account for the antioxidative behaviour are a hydroxyl group on their phenolic ring, which can terminate peroxidation chain reactions following the donation of hydrogen from the hydroxyl group (Tiidus et al., 1999, Tiidus, 1995, Sugioka et al., 1987, Persky et al., 2000). From animal studies, Tiidus and Houston (1993) showed that following strenuous exercise in female rats with high oestrogen levels, tocopherol levels were not depleted in the same way that was seen in male and sexually immature female rats from similar research (Sen et al., 1997, Bowles et al., 1991). Kendall and Eston (2002) suggested that oestrogen acts as an additional defence against RONS and could protect skeletal muscle from EIMD. Although it is not fully understood, this antioxidant action of oestrogen has also been suggested to protect cell membranes from lipid peroxidation by intercalating into the plasma membrane of a cell, altering the membrane fluidity and function (Kendall and Eston, 2002), much like the cell

stabilising actions of tocopherol and cholesterol (Wiseman et al., 1993, Tiidus, 1995, Persky et al., 2000). This could suggest that a more stable cell membrane could prevent membrane related markers of EIMD, such as CK leakage. The potential for oestrogen to act as an antioxidant provides a strong rationale that EIMD (particularly secondary EIMD) experienced following ECC exercise might be reduced in females. However, human studies tend to measure oxidative stress in the blood and not via biopsy (common in animal studies). Moreover, the oestrogen levels in animal studies are outside the natural fluctuations of oestrogen levels of eumenorrheic human females, with methods such hysterectomies commonly employed (Schneider et al., 2012, Le et al., 2018, Fulkerson et al., 2015). Therefore, given the methodological difference in animal based, sex-difference research, the extrapolation of animal data to a human population must be made with caution.

2-7.3.3 Oestrogen and exercise induced muscle damage

The notion that oestrogen can confer a protective effect against markers of EIMD has been the focus of research for some time. Long term exposure to oestrogen has been shown to increase tendon compliance (Hansen, 2016) and therefore might indirectly protect against EIMD following ECC exercise, due to an increase in mechanical buffering that can reduce muscle lengthening and EIMD markers (Guilhem et al., 2016), as discussed in section 2-6.2. Additionally, oestrogen can act in a more direct manner to alleviate EIMD. Initially, animal studies were used to manipulate endogenous levels of oestrogen to investigate the direct protective effect of oestrogen against damaging exercise. Several studies have indicated that oestrogen can significantly reduce some markers of EIMD, including attenuating strength losses, and

particularly, disruption to the muscle membrane (Schneider et al., 1999, Feng et al., 2004, Enns et al., 2008, Amelink et al., 1990, Amelink and Bar, 1986). For example, Bar et al. (1988), reported that following two hours of strenuous exercise, female rats that had been ovariectomised displayed a similar response in CK activity as male rats, and this CK response could be reduced following the supplementation of oestrogen prior to exercise. Moreover, male rats that had been supplemented with oestrogen showed a dampened CK response following exercise (Bar et al., 1988). Bar et al. (1988) suggested that the membrane stabilising effect of oestrogen was responsible for the diminished CK response, although it was also noted that the small ECC component of the exercise might explain high CK clearance rates from the blood, something that is not usually present in human studies (Newham et al., 1986). Additionally, the age at which female rats are ovariectomised might explain differences between studies. This was evidenced by Amelink and Bar (1986) rats that were ovariectomised prior to reaching sexual maturity, suffered more EIMD following exercise than rats ovariectomised after reaching sexual maturity. This suggests that chronic, high levels of oestrogen have a greater protective effect on membrane stability than acute doses. Despite these findings, there is data to suggest that there is no effect of oestrogen on the EIMD response (Warren et al., 1996, Sotiriadou et al., 2006, Moran et al., 2007). Although methodological differences, for example, only measuring the acute response to damaging exercise (Moran et al., 2007), might be the reason for the different level of protection attributable to oestrogen found between studies.

Most of the aforementioned studies have explored the theory that oestrogen stabilises cell membranes following damaging exercise, mainly through measuring blood CK

response. This method can be problematic when comparing groups due to the high variability in response and high clearance rate from the blood (Warren et al., 1996, Hyatt and Clarkson, 1998). More recent research has focussed on the potential anti-inflammatory actions of oestrogen, with data to date producing discrepant results. There is evidence to suggest that oestrogen can reduce the inflammatory response to damaging exercise (Tiidus et al., 2001, Stupka and Tiidus, 2001, Schneider et al., 1999). For example, Enns et al. (2008) reported that leukocyte infiltration was attenuated following 90 minutes of downhill running in ovariectomised female rats. In contrast, there is similar research that has found that inflammation is actually augmented by the presence of oestrogen (Tiidus, 2005, Schneider et al., 2012, Fulkerson et al., 2015, Fearing et al., 2016), and research that found contradictory or no effect of oestrogen on post exercise inflammation (Velders et al., 2012, McHale et al., 2012). A recent study by Le et al., (2018) further investigated this relationship between oestrogen and post injury inflammation in female, ovariectomise mice that received oestrogen supplementation or placebo. Results showed that the mice supplemented with oestrogen displayed augmented markers of muscle inflammation, mainly neutrophil infiltration, in comparison to the placebo group. Interestingly, no initial differences in strength were present between the two groups yet 2-6 weeks post-injury, the oestrogen supplemented mice displayed greater strength levels of 13-24%, indicating that an enhanced inflammatory response might have augmented the regenerative process, leading to faster recovery (Le et al., 2018).

As previously mentioned, there are discrepancies in the research in relation to the effect of oestrogen on post-exercise inflammation. One explanation for this could be that oestrogen concentration affects the inflammatory response in an “inverted U”

manner, whereby modest or "normal" levels of circulating oestrogen result in the most augmented inflammatory response (Straub, 2007). Tiidus (2018) commented that a "Goldilocks Zone" might exist where "normal" oestrogen levels might see the highest level of inflammation, where levels above or below "normal" might attenuate inflammation. Studies reporting an attenuated (Tiidus et al., 2001, Stupka and Tiidus, 2001, St Pierre Schneider et al., 1999, Enns et al., 2008), or (Tiidus, 2005, Schneider et al., 2012, Fulkerson et al., 2015, Fearing et al., 2016) inflammatory response following muscle injury did not track muscle strength recovery over the extended time period that Le et al. (2018) did, therefore it is uncertain whether this relationship between increased inflammation and enhanced strength recovery after 2+ weeks would have been present. Nevertheless, the findings by Le et al. (2018) provide a need for further understanding of the effect that oestrogen has on post exercise recovery and whether suppressing the inflammatory response is beneficial.

Animal studies used to investigate the relationship between oestrogen and damaging exercise provides an excellent theoretical underpinning of the mechanisms of action. Unfortunately, the common method of controlling oestrogen levels in these studies is via ovariectomy, which is not common practice in human studies. In humans, oestrogen levels can be investigated naturally (menstrual cycle fluctuations, post-menopause) or synthetically (hormone replacement or via the oral OCP). In the sports-science field, it is common to investigate the OCP and its effect on exercise recovery, therefore this will be discussed in the following section.

2-7.3.4 The oral contraceptive pill

In the United Kingdom, 83% of females of reproductive age were using contraceptives, with 42% of women surveyed being hormonal OCP users (NHS, 2018). Moreover, in a survey of 430 elite female athletes, 50% of women were currently using hormonal contraceptives, with 68% of those athletes using an OCP (Martin et al., 2018). Hormonal oral contraceptives contain synthetic versions of oestrogen and progesterone which inhibit endogenous production of both hormones, eliminating the variability of the menstrual cycle (Fleischman et al., 2010). The OCP can be taken in a cycle consisting of 21 days ingestion followed by a seven-day withdrawal/placebo phase or taken continuously across the 28-day cycle, with both methods suppressing oestrogen levels throughout the cycle (Figure 2-7), although some rises of oestrogen, to levels similar to those during the early follicular phase, can occur in the OCP-free days (Schlaff et al., 2004). The OCP works by altering the hypothalamic-pituitary-ovarian feedback loop that prevents ovarian follicles maturing (Frye, 2006), preventing ovulation and attenuating the pre-ovulation rise in oestrogen (van Heusden and Fauser, 2002). As a result, OCP using females experience significantly lower levels of oestrogen and progesterone throughout the menstrual cycle in comparison to non-OCP users (Fleischman et al., 2010, Bryant et al., 2008). Therefore, these differences in endogenous levels of oestrogen between OCP users and eumenorrheic females allow for any potential benefit of oestrogen on markers of EIMD to be investigated without any confounding sex differences.

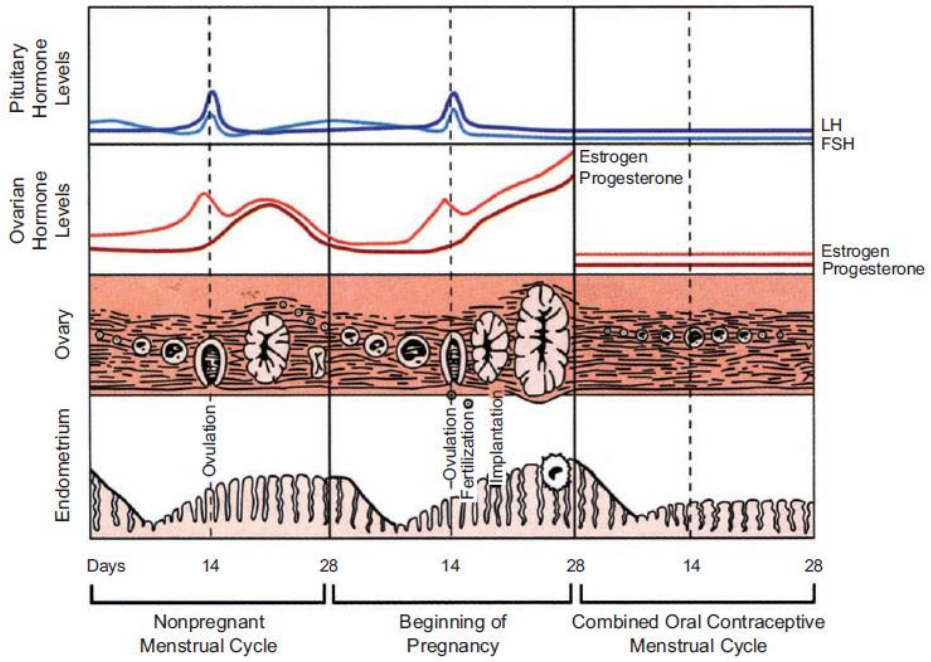

Figure 2-7 Levels of oestrogen, progesterone, and pituitary hormones, as well as depictions of the ovarian follicle and the uterine lining, in normally cycling women, pregnant women, and women taking hormonal contraceptives. LH = luteinizing hormone; FSH = follicle-stimulating hormone. Adapted from Drife (1996).

2-7.3.5 Muscle damage response in oral contraceptive pill users and eumenorrheic females

The potential for oestrogen to display a protective effect against damaging exercise has sparked an interest within sport science to further investigate this hypothesis. As mentioned previously, animal studies that have manipulated oestrogen levels have produced conflicting results on the protective effect of oestrogen (Kendall and Eston, 2002). These conflicting results are also present in human studies that have compared OCP using females and eumenorrheic females to measure the effect of circulating levels of oestrogen on markers of EIMD. For example, Thompson et al. (1997) reported no difference in muscle damage markers between OCP using females and

eumenorrheic females following a 50-minute stepping exercise. Likewise, Savage and Clarkson (2002) also found no difference in markers of EIMD following ECC exercise in the elbow flexors, with the exception of eumenorrheic females recovering more isometric strength at 48 and 72 hours than OCP using females, with this difference subsiding after 96 hours. Interestingly, Minahan et al. (2015) reported a similar pattern of strength loss as Savage and Clarkson (2002). Both OCP using females and eumenorrheic females had similar strength losses at 24 hours, but the eumenorrheic group had lost less strength at 48 hours than the OCP group. However, Minahan et al. (2015) did not measure strength losses past 48 hours, so it is difficult to see if the differences between groups would have disappeared at 72 hours, like it did in the study by Savage and Clarkson (2002). Recent data by both (Hicks et al., 2016) and Joyce et al. (2014) have both reported that eumenorrheic females experienced an attenuated CK response compared to OCP using females, following damaging exercise to the quadriceps. In addition, there were no differences in maximal strength at any time point reported by (Hicks et al., 2016) between groups, whereas strength was not measured by Joyce et al. (2014). There is a distinct lack of consistency in the data from studies that have investigated whether higher oestrogen levels attenuate EIMD markers following damaging exercise. Discrepancies in the data for (Joyce et al., 2014, Hicks et al., 2016) or against (Thompson et al., 1997, Savage and Clarkson, 2002) an oestrogen induced protective effect might be due to methodological differences between studies or the EIMD marker measured. Factors such as study length (number of follow up days), damaging protocol (e.g. maximal ECC or stepping) or EIMD markers measured (for example, Joyce et al. (2014) did not measure iMVC and

Minahan et al. (2015) only measured iMVC torque loss up until 48 h) make it difficult to identify a consistent effect.

An additional consideration is the variability of which menstrual cycle phase females were tested and the reported serum oestrogen levels between studies. For example, Thompson et al. (1997) tested eumenorrheic females during the mid-luteal phase and OCP users on day 18 of the menstrual with reported oestrogen levels of 116.0 ± 18.4 $pg \cdot ml^{-1}$ and 17.4 ± 17.1 $pg \cdot ml^{-1}$, respectively. Hicks et al. (2016) tested eumenorrheic females around ovulation (day 14) and OCP users on the 7th pill taking day, reporting oestrogen levels of 433 ± 147 and 209 ± 115 $pg \cdot ml^{-1}$, respectively. Despite Thompson et al. (1997) reporting a 6-fold + difference in oestrogen levels between OCP using females and eumenorrheic females, versus an approximate 2-fold increase between OCP users and eumenorrheic females in the study by Hicks et al. (2016), no difference in EIMD damage markers were reported, which brings into question the acute effects of high circulating oestrogen levels on muscle damage protection. However, it is possible that the participants in the study by Thompson et al. (1997) had higher progesterone levels than the participants in the study by Hicks et al. (2016), as the studies were conducted in the mid-luteal and at ovulation, respectively. The higher progesterone levels reported in the mid-luteal phase (Stricker et al., 2006) might have produced an antioestrogenic effect (Mester and Baulieu, 1984, Hsueh et al., 1975, Hodgen et al., 1994), negating the potential protective effect of oestrogen on EIMD markers. However, Thompson et al. (1997) did not report progesterone concentrations, so this remains speculative. A recent study by Anderson et al. (2017) concluded that eumenorrheic females completing a muscle damage protocol during the luteal phase of the cycle, and females during the active phase of an OCP cycle, experienced a

blunted inflammatory response and reported less soreness in comparison to eumenorrheic females in the early follicular phase (days 0-6) of the cycle. The eumenorrheic females in this study were categorised into having "high" (luteal phase) or "low" (menstrual phase) circulating oestrogen levels, as expected during the respective cycle phase (Stricker et al., 2006). However, the "high" and "low" groups produced oestrogen levels of 140.2 ± 85.7 and 115 ± 82.9 $pg \cdot ml^{-1}$, respectively in comparison to oestrogen levels of 47.5 ± 24.5 $pg \cdot ml^{-1}$ in the OCP group. With this in mind, the differences in EIMD response between groups cannot be easily attributed to oestrogen levels for three reasons. Firstly, there were no significant differences in oestrogen levels between the "high" and "low" group. Secondly, the OCP group had lower circulating oestrogen levels than both non-OCP groups, negating the comparison to the 'low' eumenorrheic group. Thirdly, progesterone levels were not measured, therefore menstrual cycle phase is more difficult to confirm (Stricker et al., 2006) and the antioestrogenic effect of progesterone cannot be ruled out (Mester and Baulieu, 1984, Hsueh et al., 1975, Hodgen et al., 1994).

These discrepancies between reported oestrogen levels and EIMD response in the study by Anderson et al. (2017), in addition to the other variables previously mentioned, such as methodological differences, highlight a need to explore this area further. A more comprehensive and consistent approach to female exercise research will provide an ability to make comparisons across studies to help the scientific community understand what effects oestrogen has on the muscle and tendon response to exercise.

2-7 THE REPEATED BOUT EFFECT

As previously established, unaccustomed exercise, particularly that involving active muscle lengthening, can result in damage to the structure of skeletal muscle that manifests in loss of muscle function, DOMS, CK release, and ultrastructural damage, amongst other responses. When performing an identical, subsequent exercise session, these markers of EIMD can be dramatically reduced. This phenomenon is known as the repeated bout effect (Nosaka and Clarkson, 1995), which can last up to six months following the initial exercise bout (Nosaka et al., 2001). Though originally investigated following two, high intensity exercise bouts (Nosaka and Clarkson, 1995), several configurations of an initial exercise bout have been shown to produce an RBE on a subsequent exercise session involving maximal ECC exercise. For example, the protective effect of the RBE has been evidenced following maximal isometric contractions at long muscle lengths (Chen et al., 2012b), electrical stimulation (Mackey et al., 2011), and low intensity/volume ECC exercise (Chen et al., 2013, Chen et al., 2012a). In addition, research has also shown that when a single limb is exercised, a protective effect is observed when the non-exercised (contralateral) limb performs a damaging bout of ECC exercise (Howatson and van Someren, 2007). However, this contralateral RBE confers protection to a lesser extent when compared to an ipsilateral condition, with the protective effect no longer evident after eight weeks (Chen et al., 2016).

Though the RBE has been the focus of many EIMD studies in recent years, the underlying mechanisms are not fully understood. The factors affecting the RBE, and the proposed underpinning mechanisms, will be discussed in detail in the following sections.

2-7.1 Mechanisms underpinning the repeated bout effect

As discussed, the magnitude of the RBE is sensitive to numerous factors. The evidence suggests that there are most likely numerous underlying mechanisms that contribute to the RBE, with reviews on the topic pertaining to four main factors; neural adaptations, a modified inflammatory response, ECM remodelling, and changes in muscle-tendon unit behaviour (McHugh et al., 1999a, McHugh, 2003, Hyldahl et al., 2017). The following sections will discuss each mechanism in detail.

2-7.1.1 Neural adaptations

Neural adaptations have been suggested to be involved in the adaptive process following damaging exercise, resulting in the RBE. Specifically, during a second bout of ECC exercise, a shift in motor unit recruitment towards low-threshold motor units has been suggested (McHugh et al., 2001). During an initial bout of ECC contractions, prior to any repeat bout, unique activation strategies are imposed which might preserve the highest threshold motor units (Enoka, 1996). During a study employing intramuscular EMG, Dartnall et al. (2011) found that after a single bout of voluntary ECC exercise, motor unit synchronisation in the elbow flexors increased by 57% for up to seven days, at which point the second exercise bout was performed. Following the second bout of exercise an RBE was present, which was supported by the attenuation in force loss after the second bout in comparison to the first. Additionally, Dartnall et al. (2011) found that following the initial bout of ECC exercise, motor unit synchronisation was increased up until the second bout was performed. Dartnall et al. (2011) suggested that the change of motor unit synchronisation after the first bout of ECC exercise was an adjustment in the motor neuron pool that resulted in the

distribution of force across a larger number of motor units, distributing the force experienced in the second bout across more muscle fibres and thus, causing less structural damage during the second exercise bout.

More recent studies have investigated the contribution of altered brain activity to the RBE. For example, Goodall et al. (2017) suggested that the RBE might be partly explained by changes in motor corticospinal drive. Specifically, a faster recovery of voluntary activation [assessed by transcranial magnetic stimulation (TMS)] was observed after a repeat bout of ECC exercise in the elbow flexors. Additional research into motor cortex adaptations and the RBE found that immediately after exercise, short-interval intracortical inhibition (SICI) was reduced following a repeated bout of ECC exercise in the dorsiflexors (Skarabot et al., 2019). Although this reduction in SICI was correlated with a reduction in iMVC at 24 hours post-exercise ($r = –0.47$, $P = 0.036$), it is likely that the reduction in SICI was due to less muscle damage as no other differences in corticospinal or spinal responses were found between bouts. Interestingly, the study by Skarabot et al. (2019), compared older and younger individuals and found a similar RBE (attenuations in loss of force and CK release) in both groups, however the reduction in SICI after the second bout was only present in the younger group. As RBE was reported in the older population despite changes in SICI, this suggests that the RBE can occur without neural adaptation. Taken together, the evidence presented suggests that a neural component to the adaptative process following ECC exercise is present and can contribute to the RBE. However, it remains unclear to what extent these neural adaptations contribute to the RBE and if these contributions are consistent across groups (e.g. old vs young) and muscles involved (e.g. elbow flexors vs dorsiflexors). What is clear is that, to an extent, a potent RBE

can exist without modulations to the neurophysiological pathway, therefore additional mechanisms must exist to explain this.

2-7.1.2 Modified inflammatory response

The inflammatory response that follows ECC exercise is essential in the regeneration process of skeletal muscle (Tidball, 2005, Chazaud, 2016), though prolonged, excess inflammation can exacerbate further degradation of tissue, known as secondary muscle damage (see section 2-4.2) (Toumi and Best, 2003, Nguyen and Tidball, 2003, Howatson and van Someren, 2008). It would therefore make sense that if excess inflammation following ECC exercise was attenuated, further disruption of the myofibrils could be avoided, leading to quicker muscle recovery, something which is typical of an RBE. Evidence of a modified inflammatory response following repeated bouts of ECC exercise was observed by Pizza et al. (1996), where lower circulating levels of neutrophils and macrophages following the second bout were recorded. However, muscle function was not measured in this study and there was no relationship found between neutrophil and macrophage levels and CK activity, leaving uncertainty over the role of this modified inflammatory response. Further studies in mice have also reported a blunted inflammatory response following ECC exercise after prior exposure to a bout of passive stretches, isometric contractions or ECC contractions (Pizza et al., 2002). Interestingly, the blunted inflammatory response after the ECC contractions was similar, regardless of whether the exercise was preceded by passive stretching, isometric contractions or ECC contractions. In addition, passive stretching, and isometric contractions did not induce any overt signs of EIMD but did induce an inflammatory response approximately half of that induced by ECC

contractions, indicating that EIMD is not necessary to induce a protective effect (Pizza et al., 2002).

This hypothesis that the RBE is driven by a blunted inflammatory repose on the second bout of exercise has seldom been explored in humans. However, Deyhle et al. (2016) employed an RBE protocol in the knee extensors to test this hypothesis. Interestingly, there was a greater inflammatory response in the second bout of ECC exercise, compared to the first, with marked increases in proinflammatory cytokines, macrophage and T-cell infiltration. There was no change in major histocompatibility complex class 1 (MHC-1) between bouts, that ruled out the authors hypothesis that MHC-1 expression was responsible for the increase in T-cell infiltration. As MHC-1 can be greatly upregulated in muscle suffering from inflammation (Englund et al., 2001, Choi et al., 2009) and has a close relationship with CD8+ T-Cells, this was a surprising result.

It has been suggested that this increased inflammatory response in the second bout of ECC exercise might increase the rate of recovery, through a more targeted response (Hyldahl et al., 2017), akin to the function of anti-bodies when repeatedly exposed to a familiar pathogen. For example, Deyhle et al. (2016) noted that although MHC-1 response did not increase in line with T-cells following a repeated bout of ECC exercise, the damaged muscle might have displayed familiar peptide sequences on MHC-1 proteins, sensitizing the T-cells to respond to muscle damage and making them more readily activatable on exposure to muscle damage (Stefanova et al., 2002). This claim, however, remains unsubstantiated and requires further investigation.

There remains contrasting evidence on the inflammatory response and the RBE, however, altered inflammatory responses following two bouts or ECC exercise have been reported. Regardless of whether an attenuated (Pizza et al., 2002, Pizza et al., 1996) or exacerbated (Deyhle et al., 2016) inflammatory response exists, it is unclear if the reduced level of EIMD is caused by these alterations in inflammatory response, or driven by them, thus providing a need for further research, particularly in human muscle damage studies.

2-7.2.3 Extracellular matrix remodelling

Much of the mechanistic research attempting to further understand the mechanisms of the RBE has given little attention to the connective tissue surrounding the contractile apparatus of the muscle. These structures, collectively known as the extracellular matrix, comprise of collagens, predominantly type I and III, glycoproteins and proteoglycans, and function to transmit forces from within the myofiber to the tendon, both longitudinally and laterally (Grounds et al., 2005). Additionally, a primary function of the ECM is to increase levels of passive tension in the MTU, to reduce the amount of mechanical strain experienced by the myofibers (Hyldahl et al., 2017). As such, this behaviour has led researchers to investigate whether the remodelling of the ECM following damaging exercise might provide a mechanistic basis for the RBE.

Transcriptomic analysis following ECC muscle actions identified that genes associated within the ECM network showed upregulated expression from pre to post-exercise (Hyldahl et al., 2011). Specifically, matricellular proteins tenascin C (TNC), cysteine-rich angiogenic inducer 61 (CYR61) and thrombospondin-1 (TSP-1) expression was increased 11.6-fold, 33.7-fold and 8.2-fold, respectively, giving a clear

indication that ECM adaptation was of significance following contraction induced injury (Hyldahl et al., 2011). RBE studies investigating ECM remodelling have found significant findings pertinent to these matricellular proteins. For example, Mackey et al. (2011), found that TNC expression was increased following a bout of damaging electrically stimulated exercise, but expression was attenuated following a repeated bout 30 days later, with such TNC behaviour being replicated following ECC exercise of the knee extensors (Hyldahl et al., 2015). Tenascin c is a facilitator of the process of cell deadhesion, which is characterised by a restructuring focal adhesions and stress fibres (Murphy-Ullrich, 2001), shifting a cell from a state of stronger adherence to weaker adherence (Schoenwaelder and Burridge, 1999). The state of deadhesion is thought to be an important part of tissue regeneration (Murphy-Ullrich, 2001), with a deficiency in TNC shown to reduce muscle generation capacity in mice (Fluck et al., 2008). Tenascin c expression positively correlates with force loss 48 h post ECC exercise (Hyldahl et al., 2015), therefore an attenuated TNC response following a repeated bout of ECC exercise could explain why attenuated strength loss is commonly experienced after ECC exercise is repeated. Although evidence of alterations to TNC expression and deadhesion activity has yet to be shown as a mechanistic underpinning of the RBE, these occurrences strengthen the hypothesis that ECM remodelling might have a prominent part to play in the RBE.

Remodelling of the ECM following ECC exercise extends beyond the initial post EIMD phase where TNC behaviour is altered. Specifically, delayed expression of collagen genes indicate that remodelling can occur for up to four weeks (Mackey et al., 2011). Following damaging exercise induced by electrical stimulation, Mackey et al. (2011) found that the gene expression of types I and III collagen were increased by

~6-9 fold, 30 days post-EIMD. Immunohistochemical staining for collagen I and III showed more intense staining patterns in the exercised leg, in comparison to the control. Taken together, the increased gene expression and intensified staining pattern provides evidence that collagen within the ECM is a site of ongoing regeneration, up to 30 days after injury. This pattern of early cellular deadhesion activity and delayed collagen activity was replicated in the knee extensors by Hyldahl et al. (2015). Interestingly, this behaviour of the initial and delayed ECM activity was unaffected by a repeated bout of exercise, despite attenuation of EIMD markers (Hyldahl et al., 2015). This indicates that the adaptation following the first bout of exercise might be responsible for the RBE seen after the second bout. Butterfield and Herzog (2006) suggest that RBE adaptations occur non-uniformly within the muscle. Therefore, another aspect to note is that changes in the collagen content of the ECM might not be ubiquitous within the injured muscle, as evidenced by increased collagen expression in the superficial but not deep regions of the muscle in animal models (Takagi et al., 2016). This, however, has yet to be displayed in human studies and warrants further investigation.

Evidence of both immediate and delayed remodelling of the ECM following damaging exercise is indicative of this being a mechanistic underpinning of the RBE. However, given that the RBE can occur following immediate preconditioning exercise (Chen et al., 2012b) non-damaging exercise (Lin et al., 2015), or on the contralateral limb (Howatson and van Someren, 2007, Chen et al., 2016) when ECM modelling is unlikely to occur, this mechanism can only partly account for the RBE.

2-7.2.4 Muscle-tendon unit behaviour

A high level of fascicle strain, leading to the overstretching of sarcomeres, is presented as a key driver of EIMD, which is predominantly explained by the 'popping sarcomere theory' (Morgan, 1990, Morgan and Proske, 2004). Following a bout of damaging ECC exercise, there is speculation that to reduce the amount of strain experienced by the muscle fibre, there is an addition of sarcomeres in series, with this presented as a possible mechanism for the RBE (Proske and Morgan, 2001). The increased sarcomere in series theory was believed to be evidenced by a shift in optimal angle towards a longer muscle length (Proske and Morgan, 2001), however this theory cannot explain the persistence of the RBE after four weeks when an optimal angle shift is no longer present (Chen et al., 2007). Moreover, the RBE has been evidenced by completing sub-maximal exercise just two days before a damaging bout of ECC exercise (Lavender and Nosaka, 2008), which given the short time between the original protective stimulus and the damaging bout, is unlikely to be indicative of myofiber remodelling. Although the addition of sarcomeres in series cannot be entirely ruled out as a mechanism underpinning the RBE, the evidence presented suggests that sarcomere remodelling cannot be solely responsible for the RBE.

Recent research has focused on muscle fascicle behaviour, with several studies unearthing a relationship between the amount of fascicle strain experienced during ECC exercise and the magnitude of EIMD experienced (Hicks et al., 2017, Guilhem et al., 2016). Moreover, research investigating repeated bouts of ECC exercise have shown alterations in MTU behaviour on repeat bouts (Penailillo et al., 2015, Lau et al., 2015). Specifically, using ultrasonography, Penailillo et al. (2015) reported a 16%

reduction in VL muscle fascicle elongation during a second bout of ECC cycling, which was associated with attenuated DOMS. Lau et al., (2015), found that the magnitude of MTJ displacement in the elbow flexors was reduced during a second bout of ECC exercise and also displayed a lower magnitude of EIMD in the second bout. These studies suggest that changes in MTU behaviour, such as an increase in tendon compliance, in contrast to the addition of sarcomeres in series (Proske and Morgan, 2001), could be responsible for the RBE. Although neither Lau et al., (2015) or Penailillo et al. (2015) directly measured tendon properties, recent evidence has also shown that the amount of fascicle strain experienced during ECC exercise can be attenuated by an increase in tendon compliance (Hicks et al., 2017, Hicks et al., 2013, Guilhem et al., 2016), although this is based on studies using a single bout of ECC exercise. To further understand the relationship between changes in MTU behaviour and the RBE, future studies must incorporate methodologies that track changes in tendon and muscle properties between repeated bouts of ECC exercise and where possible, during the ECC exercise itself.

2-7.3 Factors affecting the repeated bout effect

The repeated bout effect is often expressed by the amount that EIMD markers are reduced after the second bout of exercise, often termed the index of protection (IOP) (Chen et al., 2014). The IOP is highly sensitive and can be influenced by a number of factors pertaining to the initial bout of exercise such as the number (Burt et al., 2015), and intensity of contractions (Chen et al., 2017), muscle length (Nosaka et al.,2005a), limb involved (Chen et al., 2019), age (Chen et al., 2014), and how accustomed the participants are to the activity (Hyldahl et al., 2017). For example, manipulating the intensity of the initial bout of ECC exercise produces a proportionate level of

protection in a subsequent bout, as evidenced by Chen et al. (2007). Chen et al. (2007) compared maximal ECC exercise in the initial bout to three submaximal intensities (40%, 60% and 80%) of the elbow flexors. It was found that the protective effect was greater after the initial maximal ECC bout and that protection decreased in order of initial intensity Chen et al. (2007). Interestingly though, four repeated submaximal exercise bouts (40% max iMVC) can confer an equivocal RBE as a single, maximal ECC exercise session, thus suggesting that high levels of EIMD do not necessarily need to be present initially to induce the RBE (Chen et al., 2010). It is apparent that the magnitude of the RBE is sensitive to the intensity of the initial exercise bout, however the two studies mentioned by Chen et al. (2007, 2010) both used an equal number of repetitions in the high and low intensity protocols and were not matched for mechanical work. Recently, Mavropalias et al. (2020), implemented a work matched high or low intensity ECC cycling protocol (20% peak power output (PPO) and 5% PPO, respectively) for the initial RBE exercise session. Both high and low intensity groups repeated the exercise protocol 14 days later at the high intensity (20% PPO) and it was found that there were no differences between groups for post-exercise muscle function. There were group differences in DOMS, with the group who performed low intensity exercise in the first bout reporting higher levels of soreness post bout two (Mavropalias et al., 2020). Given the evidence presented, it appears that when ECC exercise is matched for the amount of mechanical work performed, intensity has no effect on the RBE in relation to muscle function but might attenuate soreness when the initial bout is more intense. It should be noted that the study by Mavropalias et al. (2020) employed submaximal exercise (ECC cycling), which has been shown to induce only modest levels of EIMD Penailillo et al. (2015). Therefore,

RBE studies that use maximal ECC protocols compared against work matched submaximal ECC exercise should be explored to further understand the effect of intensity on the magnitude of the RBE.

The RBE is also sensitive to contraction type and does not require an ECC action in the first exercise bout. For example, 30 iMVCs lasting 5 s with 45 s between repetitions, prior to 30 maximal ECCs of the elbow flexors three weeks later, conferred a protective effect (Chen et al., 2012a). However, only iMVCs at a long muscle length conferred this protection, as in the same study, no protective effect was reported for iMVCs at short muscle length (Chen et al., 2012b). This effect of iMVCs at long muscle lengths has been seen after just two contractions in the elbow flexors, however no RBE was seen after seven days (Chen et al., 2013). An additional factor that can influence the magnitude of the RBE is contraction velocity, although mixed evidence exists. Chapman et al. (2011) found that a bout of maximal ECC actions of the elbow flexors at a slow velocity, conferred protection against EIMD after a bout of fast velocity maximal ECC actions in the same arm, three weeks later. In contrast, Barss et al. (2014) found that maximal ECC actions of the elbow flexors only produced an RBE when contraction velocity was kept constant between the two bouts; fast velocity contractions did not reduce markers of EIMD after the second bout consisting of slow velocity contractions, and *vice versa*. Interestingly, a recent study, Barreto et al. (2019) combined the use of isometric pre-conditioning (10 iMVCs at long muscle length) two days prior to either fast or slow velocity maximal ECC exercise of the elbow flexors. This study found that iMVCs two days prior to maximal ECC exercise produced a protective effect, irrespective of contraction velocity. These results have important implications for the design of research investigating EIMD and the RBE.

The data presented suggests that there are several factors that can protect the muscle from a damaging bout of ECC exercise. These can be generally summarised to conclude a protective effect can be induced by; 1) identical bouts of maximal ECC exercise, 2) non- or low-damaging, sub-maximal ECC exercise, 3) isometric exercises at long muscle lengths, 4) maximal ECC on the contralateral limb and 5) low volume, maximal ECC exercise. It is likely that many combinations of these exercises might induce an RBE.

2-8 SEX DIFFERENCES IN EXERCISE INDUCED MUSCLE DAMAGE AND THE REPEATED BOUT EFFECT

Study of the varying responses of men and women to ECC exercise has increased over the last decade, however there is still no definitive answer to whether a sex difference exists. Early animal studies reported that males display a higher susceptibility to EIMD (St Pierre Schneider et al., 1999, Komulainen et al., 1999, Clarkson and Hubal, 2002). In human studies, there is less clarity as to whether there is a sex difference in EIMD response between the sexes. Evidence exists supporting (Wolf et al., 2012, Sewright et al., 2008, Oosthuyse and Bosch, 2017, Joyce et al., 2014, Hicks et al., 2016, Fredsted et al., 2008, Amorim et al., 2014) or refuting (Stupka et al., 2000, Sayers and Clarkson, 2001, Rinard et al., 2000, Dannecker et al., 2012) a sex difference in EIMD response.

2-8.1 Sex differences in exercise induced muscle damage following a single bout of eccentric exercise

Studies investigating sex differences that use direct measures of EIMD are uncommon, with only Stupka et al. (2000) presenting such data, reporting no sex difference in Z-

line streaming following ECC exercise in the lower limb. Therefore, the use of indirect markers of EIMD are generally implemented to detect sex differences in the muscle damage response, which can produce varying results dependent on the measure used. Strength loss, as measured by iMVC, is a valid marker of EIMD (Warren et al., 1999, Nosaka et al., 2006) and when implemented, has produced contrasting results in the EIMD sex-difference literature. For example, following an ECC step protocol, Fredsted et al. (2008), reported that females experienced greater losses in knee extensor strength at 24, 48 and 72 hours post-exercise than males. Moreover, Sewright et al. (2008) reported a greater loss in iMVC force immediately after ECC exercise of the elbow flexors but not at any other timepoint, which could be an indicator of fatigue and not EIMD (Walsh et al., 2004). Minahan et al. (2015) reported losses in quadricep iMVC force immediately after and 24 h post exercise in males, OCP-using females and eumenorrheic females. However, after 48 hours, only eumenorrheic females showed no further decline in strength loss whereas the males and OCP using females did, with the authors suggesting that oestrogen facilitated the enhanced recovery. Other studies have shown no sex differences in strength loss following ECC exercise of the elbow flexors (Sayers and Clarkson 2001, Savage and Clarkson 2002) or knee extensors (Hicks et al., 2016). The contrasting evidence in relation to sex differences in strength loss is difficult to explain when different limbs are being tested, especially when upper limbs are more susceptible to damage than lower limbs (Saka et al., 2009, Chen et al., 2019, Chen et al., 2011). Nevertheless, in contrast to the animal data, the larger strength loss reported by Fredsted et al. (2008) was attributed to the female participants exercising at a higher relative intensity than males. Although this cannot be confirmed due to total work not being reported, a recent meta-analysis suggests that

when strength loss in normalised for ECC work, no sex differences in strength losses are present (Morawetz et al., 2019). Overall, it appears that a distinct conclusion cannot be drawn as to whether a consistent difference in strength loss exists following ECC exercise between males and females.

Compared to males, females have higher circulating levels of oestrogen (Velle, 1966, Tepperman, 1987, Kendall and Eston, 2002) and with oestrogen reported to have a predominant cell membrane stabilising effect (Kendall and Eston, 2002), it would be prudent to include measures of membrane stability in sex related EIMD studies. Indeed, several studies have reported the CK response to ECC exercise, with strong evidence suggesting that females demonstrate an attenuated CK response in comparison to males in the knee extensors (Joyce et al., 2014, Hicke et al., 2016) and the elbow flexors (Sewright et al., 2008). In contrast, Stupka et al. (2000) did not report a sex difference in CK following ECC knee extensor exercise, but did report an attenuated inflammatory response, determined by circulating leukocyte concentration, in females, which they attributed to the membrane stabilising effect of oestrogen. This is surprising considering that the membrane stabilising effect of oestrogen is also thought to be the mechanism of attenuated CK release following ECC exercise (Kendall and Eston, 2002). This anomaly could potentially be explained by the time course of sampling as Stupka et al. (2000) measured CK at 48 and 144 hours post-exercise, when CK reportedly peaks at 72-96 hours (Saka et al., 2009, Clarkson et al., 1992), thereby giving a false account of true CK peak values. It might be considered that higher CK values in males is due to higher volumes of exercised muscle, however, in a recent meta-analysis, Morawetz et al. (2019) reported that despite normalising CK data to muscle CSA, males still reported higher peak levels of CK, giving weight to

the theory that oestrogen acts to stabilise cell membranes and attenuates CK release (Kendall and Eston, 2002). Moreover, after CK response being normalised to quadricep CSA, Hicks et al. (2016) found that males exhibited a greater CK response than females following 6 sets of 12 maximal ECC knee extensions. Despite this difference in CK, no sex differences in strength loss were found (Hicks et al., 2016) making it difficult to draw any solid EIMD sex-difference conclusions based on this evidence.

The evidence presented shows that there is contrasting evidence of a differing EIMD response between males and females when using post-exercise strength loss as a measure. There appears to be more consistent evidence that shows an attenuated CK response in females compared to males, however, given that a direct relationship between CK and EIMD is lacking (Stupka et al., 2000, Stupka and Tiidus, 2001) and CK sex differences can be present in the absence of strength loss differences (Hicks et al., 2016), it remains unclear whether males and females respond differently to damaging ECC exercise.

2-8.2 Sex differences in exercise induced muscle damage following repeated bouts of eccentric exercise

Repeated bouts of ECC exercise typically result in reduced markers of EIMD after the second bout (Nosaka and Clarkson, 1995). The magnitude of RBE is determined by a number of factors (contraction intensity, muscle length, contraction velocity etc.) as discussed earlier (section 2-6). However, much less is known on how sex influences the magnitude of the RBE. Although several RBE studies have included a mix of male and female participants (McHugh and Pasiakos, 2004, Deyhle et al., 2016, Cleary et al., 2002, Black and McCully, 2008), to our knowledge, only Stupka et al. (2001)

directly assessed sex differences in the magnitude of the RBE. Eight males and eight females performed three sets of 12 unilateral leg press exercises followed by 100 ECC leg extensions. No RBE differences between males and females were found with the exception of an increase in neutrophil infiltration in women, 24 hours after the second bout. As neutrophil infiltration is relatively rapid following damaging exercise (Fielding et al., 2000), it is possible that neutrophil levels in male participants had returned to baseline levels after the second exercise bout. Additionally, alterations to the time course of the inflammatory response have been observed in males following repeated exercise bouts (Smith et al., 1998), which makes it plausible that peak neutrophil infiltration in the male participants might have been missed. Whether a time course difference following repeated bouts of exercise exists for females remains unclear.

Typically, studies investigating the RBE in female only populations (Nikolaidis et al., 2007, Muanjai et al., 2019, Lin et al., 2017, Brown et al., 2016) have observed responses typical of the RBE (attenuated strength loss, CK release and DOMS) demonstrated in male populations. However, given the heterogeneity of methodologies between studies, it is difficult to ascertain whether the RBE response of males and females is equivalent. Even Stupka et al. (2001), who implemented the same methodology between male and female groups, acknowledged that due to the small group numbers (n = 8 per group), the lack of statistical power meant that the sex difference in the inflammatory response should be interpreted with caution. Therefore, to understand the RBE response following repeated ECC exercise, studies need to be conducted that implement the same study protocol between males and females. Moreover, no study to date has investigated the effect of the OCP on the magnitude of

the RBE. Given the potential for oestrogen to influence the muscle damage response (Kendall and Eston, 2002), it would be prudent to implement OCP use as factor into future study design.

2-9 AIMS

In light of the aforementioned research, the main aim of this thesis was to use 2D B-mode ultrasonography to further understand the properties and behaviour of the patella tendon and *vastus lateralis* in males and females and how these properties contribute to the EIMD response to ECC exercise and the magnitude of the repeated bout effect. To achieve this aim, the thesis will consist of three studies, the aims of which are outlined below.

Study 1: The reliability and validity of measuring patellar tendon cross-sectional area using 2D B-mode ultrasonography and magnetic resonance imaging.

Aims: 1) Determine the agreement between US and magnetic resonance imaging (MRI) measures of PT CSA for two independent raters 2) determine the within-day, inter- and intra-rater reliability for US and MRI measures of PT CSA and, 3) determine the between-day, intra-rater reliability of US measures of PT CSA.

Study 2: Test-retest reliability of measuring patella tendon properties and muscle fascicle length changes in the vastus lateralis during maximal eccentric exercise, using 2d b-mode ultrasonography.

Aims: Determine the reliability of using US to quantify PT properties at rest and during maximal isometric exercise, and VL properties at rest and during maximal ECC exercise.

Study 3: Sex differences in exercise induced muscle damage and the repeated bout effect following maximum, single limb, knee extension exercise

Aims: To assess whether adaptations in MTU behaviour during maximal lengthening contractions can explain the RBE, and whether these adaptations differ between males, eumenorrheic females, and females taking the OCP. Additionally, to investigate the potential relationships between MTU behaviour and the magnitude of EIMD and the RBE.

CHAPTER 3 THE RELIABILITY AND VALIDITY OF MEASURING PATELLAR TENDON CROSS-SECTIONAL AREA USING 2D B-MODE ULTRASONOGRAPHY AND MAGNETIC RESONANCE IMAGING

3-1 INTRODUCTION

The human patellar tendon (PT) plays a crucial role in locomotion by transmitting force from the quadriceps muscle group to the tibia. Tendon is a viscoelastic tissue and will deform under loading (Magnusson et al., 2008), with the degree of loading corresponding to the structural properties of the tissue (Maganaris and Paul, 1999). These structural properties, such as tendon stiffness and YM, determine the compliance of the tendon, which in turn might mediate the magnitude of EIMD experienced during ECC exercise. Specifically, tendons can act as a mechanical buffer during high force ECC contractions, reducing the amount of muscle fascicle strain during such movements (Hicks et al., 2013) and potentially alleviating the magnitude of EIMD experienced (Guilhem et al., 2016, Hicks et al., 2017).

To determine the role of the PT and its compliance in the mechanical buffering of force, accurate and reliable measurements of these properties and behaviours must be performed. Specifically, tendon CSA is an important measure needed to estimate the material properties of the PT, such as YM.

Magnetic resonance imaging is a technique that uses the chemical and magnetic environment surrounding unpaired hydrogens, and their magnetic properties, to produce tissue images (Berger, 2002). The strong magnetic environment produced by an MRI machine forces the protons in the body to behave like bar magnets and align with the magnetic field (Read and Peduto, 2000). The protons are then excited to a higher energy state by an electromagnetic pulse at the natural or radio frequency (RFr) of the proton, which when terminated, causes the proton to return to alignment and emit a lower RFr. This contrasting RFr energy can be detected and built to form an

image, with the different tissues (lipid, bone, tendon etc.) detectable by altered proton relaxation rates, which is what produces excellent image contrasting (Read and Peduto, 2000). Several studies have validated the accuracy of MRI in measuring tendon properties (Sonin et al., 1996, Carrino et al., 1997, Berthoty et al., 1989), with MRI considered the 'gold standard' of tendon property measurement and routinely used as a validation tool against other measurement techniques (Kruse et al., 2017, Bohm et al., 2016).

Another form of *in vivo* measurement technique for tendon properties is 2D B-mode ultrasound imaging, which is an imaging technique that builds up a cross-sectional display of soft tissue using the SONAR principle. Throughout this thesis, 2D B-mode ultrasound imaging will be referred to solely as "ultrasound imaging", unless stated otherwise. For tendon imaging, this process requires a transducer (typically 5-12 MHz) placed on the skin, which transmits ultrasonic waves into the tissue before returning to the same transducer, where the depth and the brightness are determined and plotted (Read and Peduto, 2000). The ultrasound image is constructed based on numerous acoustic and structural interactions, with specific effects on beam interference, attenuation, refraction, and reflection, resulting in the possibility of distinction between tissue (Read and Peduto, 2000). Ultrasound technology is commonly used in research settings to assess tendon size and to measure the tendon's response to loading and healing (Thoirs and Childs, 2018). Ultrasound is an affordable, time efficient, portable and non-invasive imaging technique, which makes it an attractive tool in musculoskeletal research. As with all diagnostic tools, US is not without its limitations and a number of considerations need to be taken into account when the method is used. For example, US accuracy and reliability are dependent on

a number of factors such as operator experience (Wallwork et al., 2007), non-standardised imaging protocols, and slight variations in transducer positioning (Gellhorn and Carlson, 2013). To have confidence in the values of tendon properties calculated from US derived dimensions, measurement error must be understood.

The reliability and accuracy of US tendon measures is conflicted within the literature (McAuliffe et al., 2017, Gellhorn and Carlson, 2013). For example, Reeves et al. (2003), reported that between-day, intra-rater reliability showed strong agreement between measures of PT CSA. In addition, Gellhorn and Carlson (2013) found that both experienced and inexperienced US operators showed high levels of inter- and intra-rater reliability, and inter-machine reliability for measuring PT CSA, when strict scanning protocols were used. In contrast, Bohm et al., (2016) and Ekizos et al. (2013) found that US measures of AT CSA and PT CSA, respectively, were unreliable, with Ekizos et al. (2013) attributing this to poor definition of tendon borders. Interestingly, Kruse et al. (2017) reported that intra-rater US measures of AT were reliable but not interchangeable with MRI measures, as US underestimated AT CSA by ~5.5%. The agreement of US and MRI measures of tendon CSA has also produced contrasting results in the literature. Similar to Kruse et al. (2017), Bohm et al., (2016) found that US measures of the AT underestimated CSA by ~19%. In contrast, recent research by Stenroth et al. (2019) found that when highly standardized measurement protocols are implemented and an experienced rater is used, agreement between US and MRI measures of both the AT and PT is high. One factor that might explain the discrepancies between MRI and US tendon analysis might be that MRI analysis overestimates CSA because of the inclusion of the paratenon, the areolar tissue between the tendon and its sheath, which is commonly excluded during US analysis

(Kruse et al., 2017). Additionally, the method used to digitise and analyse images might affect the agreement between US and MRI tendon analysis. For example, Kruse et al. (2017) employed a manual tracing technique for US images of the AT and a threshold cut-off method for MRI analysis and found poor agreement between the two measurement methods. Stenroth et al. (2019) used the same method of image digitisation and analysis for both US and MRI images and reported good agreement between the methods, but only when the rater was experienced (> five years of musculoskeletal imaging and segmentation (Stenroth et al., 2019). Collectively, these data suggested that the reliability and accuracy of US and MRI measures of tendon CSA are inconsistent and require further investigation. Of particular importance to this project, one key factor appears to be the experience of the US operator (McAuliffe et al., 2017, Hayes et al., 2019), and therefore it is prudent to assess the accuracy and reliability of the US operators in the present study and the subsequent studies in this thesis. Therefore the aims of this present study are threefold: 1) determine the agreement between US and MRI measures of PT CSA for two independent raters, 2) determine the within-day, inter- and intra-rater reliability for US and MRI measures of PT CSA, and 3) determine the between-day, intra-rater reliability of US measures of PT CSA. In relation to the overall aim of this thesis, this study will provide confidence that the calculations used to determine patella tendon properties, and any changes in such properties resulting from exercise intervention, are based on accurate measures of PT CSA.

3-2 METHODS

3-2.1 Participants

Nineteen participants, ten females and nine males, participated in the study (age: 25 ± 6 years; stature: 1.71 ± 0.10 m; mass: 71.3 ± 12.5 kg). Participants completed a pre-test questionnaire and were only included in the study if they had no neuromuscular or musculoskeletal impairments in the lower limbs within the last six months. Contraindications for MRI included cardiac pacemaker, metal objects in the body (such as aneurysm clips or a programmable shunt in the brain), joint prosthesis, bone fixation devices, and pregnancy. Institutional ethical approval was received from the Northumbria University Faculty of Health & Life Sciences Ethics committee in accordance with the *Declaration of Helsinki*. Participants were supplied with a participant information sheet, detailing the purpose of the study and provided written consent before participating (Appendix 1).

3-2.2 Experimental design

Participants were requested to visit the laboratory on three occasions. The first session was the imaging of the PT using MRI, the second and third visits were the imaging of the PT using US, separated by three days, with the second session one week after the first. In session one, two MRI scans of the PT were performed, separated by a short interval, where the participant was removed from the MRI scanner before being repositioned within the MRI scanner and scanned again. In sessions two and three, two raters each performed two US scans of the PT on the same leg. The participant was then removed from the scanning position, before being repositioned and US scans performed again, resulting in four US scans per rater, per visit (eight US scans in total).

Rater 1 was considered less experienced, however, training in image acquisition using US, and image digitisation and analysis was provided in depth before the study by Rater 2, who was experienced over a five-year period in musculoskeletal radiography. Imaging was performed at the same time of day in each session to remove diurnal effects on tendon size (Stenroth et al., 2019). Prior to each visit, participants were asked to refrain from strenuous lower body exercise for 48 hours to reduce possible deformations in the PT structure due to fluid ecchymosis.

3-2.3 Procedures

MRI Examinations

Participants attended Newcastle Clinic for the MRI examinations. Prior to scanning, measures of stature and mass were recorded using a portable stadiometer (Seca model 213; Seca, Hamburg, Germany) and digital scales (Seca model 813; Seca, Hamburg, Germany) respectively. Participants were placed in an open MRI device (GE Ovation 0.35 T open MRI scanner, GE Healthcare, Little Chalfont, UK) in a left decubitus position, with the hip and knee flexed to 85° and 90° (0° = full extension), respectively (Figure 3-1a), confirmed using a goniometer (Idass 360° goniometer; Idass, Cornwall, UK). This positioning was chosen to mirror the hip and knee angles of participants during the US measurements in sessions two and three. The right knee was scanned for all participants. The scan procedure was performed by a qualified radiographer at Newcastle Clinic after positioning of the knee had been confirmed by the researcher.

The MRI scan procedure was divided into three sections; a localiser scan to confirm correct field of view, a sagittal plane scan (spin echo T1, TR/TE 500/24, field of view 20 ×15 cm, slice thickness 4 mm, spacing between slices 0 mm, 03:45 mins scan time)

followed by an axial scan (spin echo T1, TR/TE 450/24, field of view 20 x 15cm, slice thickness 4 mm, spacing between slices 0 mm, 05:31 mins scan time). The scanning procedure was repeated after the participant was repositioned, to assess for reproducibility.

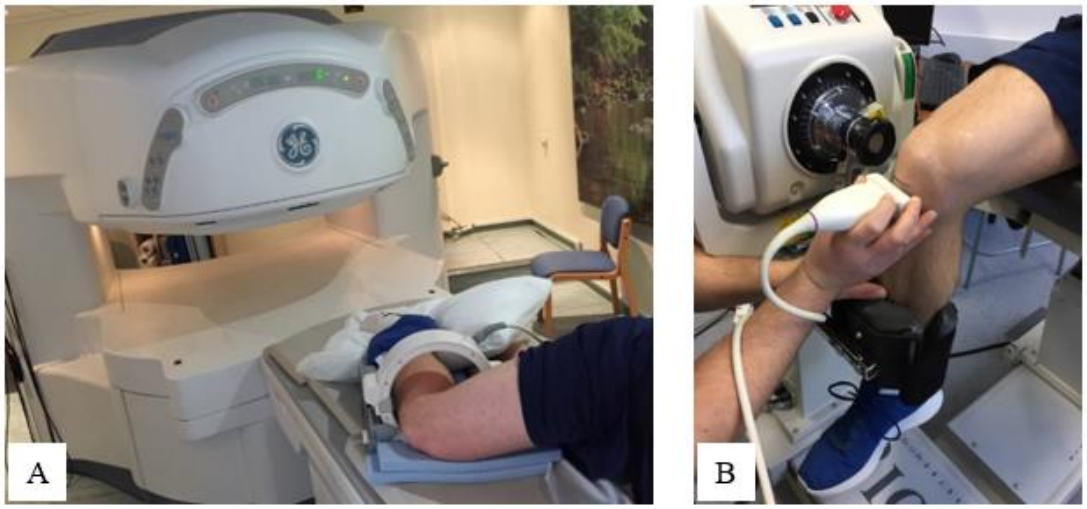

Figure 3-1 Scanning position prior to the MRI scan (A) and scanning position for US imaging (B).

Ultrasound Examinations

Participants were positioned in an isokinetic dynamometer (System 4 Pro, Biodex Medical Systems Inc., NY, USA) in a seated position, with the hip and knee flexed to 85° and 90° (0° = full extension), respectively (Figure 3-1b). A real-time B-mode ultrasound (HDI 5000 SonoCT, Philips, Amsterdam, Netherlands) was used to assess PT CSA and PT length. Sagittal images of the PT were obtained using an US probe (7.5 MHz linear array probe, 55 mm width) to locate the apex of the patellar and the tibial tuberosity, with marks placed on the skin at each site. US images were enhanced using a conductive gel (Aquasonic 100, Parker Laboratories Inc., Fairfield, NJ, USA). The distance between the two sites was measured via an inextensible anthropometric

tape measure and taken as PT length. PT CSA was measured in the axial plane at 25% (proximal), 50% (mid) and 75% (distal) of PT length, with the scan locations clearly marked on the skin. Ultrasound images were captured live using image acquisition software (AVer Media Capture Studio, AVer Media Technologies, New Taipei City, Taiwan) and analysed offline. PT CSA images were obtained by two US operators. Within each US session, the participant was removed from the dynamometer and the scan location marks were removed from the skin before being repositioned. The procedure was then repeated to allow for within session reliability to be assessed.

MRI image analysis

Sagittal MRI images were used to locate the apex of the patellar and the tibial tuberosity, to ensure consistency of anatomical landmarks used to determine PT length during US examinations. From the sagittal MRI images, the corresponding axial image could be identified. The number of images between the axial image for the apex of the patellar and the tibial tuberosity was used to determine the PT CSA image at 25, 50 and 75% of PT length. For example, if 12 images lay between the apex of the patellar and the tibial tuberosity, images 3, 6 and 9 would be analysed for 25, 50 and 75% PT length, respectively. Should the appropriate point lay between two images, the image towards the proximal region of the PT was analysed.

MRI images were exported and analysed by digitising software (ImageJ 1.45; National Institutes of Health, Bethesda, MD, USA). Images were firstly converted to 32-bit greyscale. An adjustable threshold cut-off method was used to determine PT borders (Kruse et al.,2017). The threshold was adjusted until the smallest natural appearance of the PT was achieved (Figure 3-2a). Patellar tendon CSA was then calculated by the

software. Both raters performed this sequence twice for each image with the mean PT CSA recorded for further analysis. All images were independently blinded and randomised for both raters prior to analysis to reduce researcher bias.

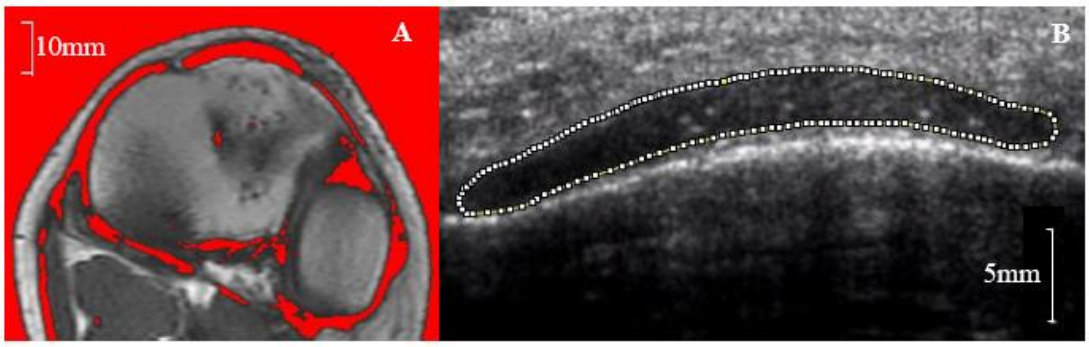

Figure 3-2 Axial MRI image showing the automatically outlined PT CSA (A) and the corresponding axial US image showing the manually outlined PT CSA (B) using imageJ software (ImageJ 1.45; National Institutes of Health, Bethesda, MD, USA).

Ultrasound image analysis

Ultrasound videos were exported to video editing software (Adobe Premier Elements version 15, Adobe, CA, USA) for frame-by-frame analysis. The images at the appropriate PT CSA location were manually assessed, before being exported for analysis in imageJ software. The tendon border was then manually outlined, and the CSA was calculated (Figure 3-2b). Each rater manually analysed each image twice, with the mean PT CSA used for further analysis. All US images were independently blinded before both raters analysed all images in a randomised order to prevent the possibility of systematic error due to recalling previous analysis.

3-2.4 Statistical analysis

Data are presented as mean ± SD. The level of significance was set to $\alpha = 0.05$. Data were analysed using a published spreadsheet (Hopkins, 2015) in Microsoft Excel (Microsoft Excel 2016, Microsoft, Washington D.C., USA) as follows:

MRI vs US comparison. Agreement between MRI and US was assessed via linear regression for each rater individually, and the collapsed scores between both raters. Pearson correlation coefficients and the standard error of the estimate (SEE) were calculated for PT CSA values. Additionally, paired sample t-tests were used to assess for systematic error.

MRI and US reliability. Relative reliability of MRI and US measurements was assessed using intraclass correlation coefficients ($ICC_{3,1}$), while absolute reliability was assessed by calculating typical error (TE) (95% confidence intervals) expressed as raw units and as a coefficient of variation (CV, %). The standard error of measurement (SEM) was calculated as the square root of the mean square error from a one-way analysis of variance (ANOVA) (Stenroth et al., 2019). Paired sample t-tests were implemented to assess for systematic error (Dragiev et al., 2011). Reliability via ICC was interpreted by the following: ICC 0.5 – 0.75, moderately reliable, ICC 0.75-0.9, good reliability, ICC > 0.9, excellent reliability (Koo and Li, 2016). Reliability was assessed for the proximal, mid and distal PT CSA images individually, in addition to the mean of all three PT CSA scores.

Inter- Intra-rater reliability. Within-day, intra-rater reliability was assessed for MRI and US images by comparing the PT CSA scores between the first and second image in each session. Between-day, intra-rater reliability was assessed for US images by comparing the PT CSA scores of the first scan for each rater (before the participant was repositioned) for each visit. Within-day, inter-rater reliability for MRI and US images was assessed by comparing the PT CSA scores of rater 1 and rater 2 (before the participant was repositioned) during visit 1. Relative reliability of MRI and US

measurements was assessed using $ICC_{3,1}$, while absolute reliability was assessed by calculating SEM (described earlier) and calculating TE (95% confidence intervals) expressed as raw units and as a coefficient of variation (CV, %). Paired sample t-tests were implemented to assess for systematic error (Dragiev et al., 2011).

3-3 RESULTS

Magnetic resonance imaging vs Ultrasonography

Mean ± SD PT CSA scores are presented in Table 3-1. Individual data points for MRI and US measures of PT CSA are displayed in Figure 3-3. No systematic differences were present when proximal, mid and distal values were averaged for both raters individually and when values were averaged across both raters. For rater 1, there was evidence of a small systematic bias, as US underestimated MRI PT CSA by 2.6 mm^2 (p = 0.017) and 5.3 mm^2 (p = 0.008) for proximal and distal measurements, respectively, compared to MRI. For rater 1, SEE (raw units) ranged from 4.0 - 8.1 mm^2 for proximal, mid and distal measures and 3.3 mm^2 when scores were combined. Pearson's r ranged from 0.88 – 0.96 for location specific measures and 0.97 for combined scores. For rater 2, SEE ranged from 4.6 – 7.3 mm^2 for location specific measures and 2.6 mm^2 when scores were combined. Pearson's r ranged from 0.89 - 0.95 for location specific measures and 0.98 when scores were combined. Collapsed scores for raters 1 and 2 produced SEEs that ranged from 3.8 – 4.7 mm^2 for location specific measures and 2.4 mm^2 when scores were combined. Pearson's r ranged from 0.95 – 0.97 for location specific measures and 0.98 for combined scores.

Within-day intra-rater reliability

Mean ± SD PT CSA scores for within-day, MRI and US analysis are presented in Table 3-2. The individual within-day PT CSA scores for rater 1 and rater 2 are displayed in Figure 3-4a and Figure 3-4b for US and MRI measurements, respectively. For rater 2, US analysis overestimated PT CSA by 1.4 mm^2 in measure two compared with measure one (p = 0.028) for the proximal PT CSA. No other systematic differences between the first and second measure were found for US or MRI analysis. TE (raw units) for rater 1 US analysis ranged from 1.5 - 2.7 mm^2 for location specific measures and 1.4 mm^2 for combined scores. In comparison, TE for rater 1 MRI analysis range from 4.6 – 6.7 mm^2 for location specific measures and was 3.6 mm^2 for combined scores. TE for rater 2 US analysis ranged from 1.8 – 3.0 mm^2 for location specific analysis and 1.3 mm^2 for combined scores. In comparison, TE for rater 2 MRI analysis ranged from 4.5 – 6.7 mm^2 for location specific analysis and 3.0 mm^2 for combined scores. Within-day measures were good (ICC $\geq$ 0.81) for rater 1 distal MRI, rater 2 proximal MRI and distal MRI analysis. All other within-day measures were considered excellent (ICC $\geq$ 0.91).

Table 3-1 Agreement between US and MRI measures of patellar tendon cross-sectional area

	MRI (mm^2)	US (mm^2)	Bias (95% CI)	*P*	SEE	PCC
Rater 1						
Proximal	84.0 ± 14.7	81.4 ± 12.5	–2.6 (–4.6 - –0.5)	0.017*	4.0	0.96
Mid	87.8 ± 14.9	82.6 ± 14.0	–5.3 (–9.0 - –1.5)	0.008*	7.9	0.86
Distal	91.1 ± 16.6	91.3 ± 14.2	0.2 (–3.6 - 4.0)	0.905	8.1	0.88
Mean†	87.6 ± 14.3	86.7 ± 12.6	–0.9 (–2.6 - 0.8)	0.278	3.3	0.97
Rater 2						
Proximal	84.5 ± 14.0	83.5 ± 14.3	–0.9 (–3.1 - 1.3)	0.395	4.6	0.95
Mid	88.3 ± 16.2	89.3 ± 14.7	1.1 (–1.8 - 3.9)	0.431	6.1	0.93
Distal	90.8 ± 15.4	91.1 ± 14.1	0.3 (–3.1 - 3.7)	0.863	7.3	0.89
Mean†	87.8 ± 13.9	88.0 ± 14.0	0.2 (–1.1 - 1.4)	0.793	2.6	0.98
Combined						
Proximal	84.2 ± 14.3	82.5 ± 13.2	–1.7 (–3.5 - 0.1)	0.055	3.8	0.97
Mid	88.0 ± 15.5	88.4 ± 13.8	0.4 (–1.5 - 2.3)	0.673	3.9	0.97
Distal	91.5 ± 14.6	91.2 ± 13.9	–0.3 (–2.5 - 1.9)	0.785	4.7	0.95
Mean†	87.4 ± 13.2	87.7 ± 14.0	0.4 (–0.9 - 1.6)	0.541	2.4	0.98

Data are presented as mean ± SD. MRI = magnetic resonance imaging; US = ultrasound imaging; CI = confidence interval; *P* = paired samples t-test; SEE = standard error of the estimate; PCC = Pearson correlation coefficient; † = mean score of the proximal, mid and distal scores; * = significantly different between US and MRI

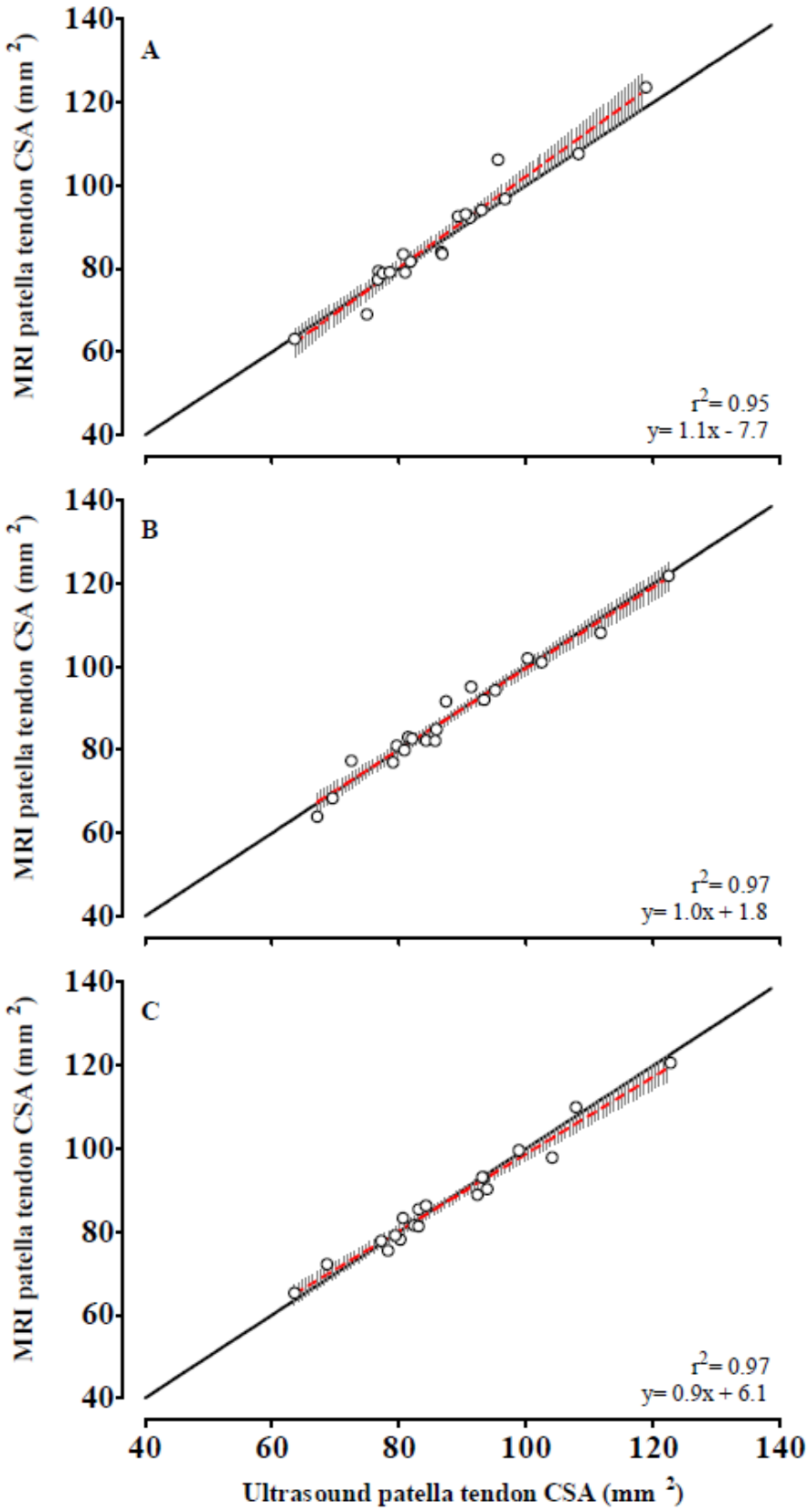

Figure 3-3. Individual data points for the agreement between estimations of patellar tendon cross-sectional area using ultrasound and MRI. Panel A = Rater 1, Panel B = Rater 2, Panel B = Rater 1 and 2 combined. The solid line represents the line of equality. The dashed red line denotes the regression line with corresponding 95% CIs represented in the grey hashed area. CSA = cross sectional area. MRI = magnetic resonance imaging.

Between-day intra-rater reliability

The PT CSA scores for between-day US analysis are presented in Table 3-3. The individual, between-day PT CSA scores for rater 1 and rater 2 are displayed in Figure 3-4c for US measurements. No systematic differences were present between visits for either rater. TE for rater 1 ranged from 3.2 – 3.5 mm^2 for location specific measures and was 2.3 mm^2 for combined scores. TE for rater 2 ranged from 2.6 – 3.7mm^2 for location specific measures and was 1.6 mm^2 for combined scores. All between-day measures were considered excellent (ICC $\geq$ 0.94).

Within-day inter-rater reliability

Patella tendon CSA scores for within-day US and MRI analysis are presented in Table 3-4. The individual, within-day PT CSA inter-rater scores are displayed in Figure 3-4d for MRI and US measurements. No systematic differences were found between raters for MRI or US analysis. Typical error for US analysis ranged from 3.3 - 4.3 mm^2 for location specific measures and was 2.4 mm^2 for combined scores. Typical error for MRI analysis ranged from 2.2 - 2.8 mm^2 for location specific measures and was 1.5 mm^2 for combined scores. All within-day, inter-rater scores were considered excellent (ICC $\geq$ 0.92).

Table 3-2 Within-day intra-rater reliability for estimates of patellar tendon cross-sectional area using US and MRI

	Measure 1 (mm^2)	Measure 2 (mm^2)	Bias (95% CI)	*P*	TE (95% CI)	CV (95% CI)	ICC	SEM	SEM%
Rater 1 US									
Proximal	81.4 ± 12.5	82.6 ± 14.0	1.2 (–0.5 - 2.9)	0.158	2.5 (1.9 - 3.7)	2.6 (1.9 - 3.8)	0.97	3.6	4.2
Mid	87.5 ± 13.5	87.8 ± 14.1	0.3 (–0.8 - 1.2)	0.606	1.5 (1.1 - 2.2)	1.7 (1.3 - 2.5)	0.99	0.8	0.8
Distal	91.3 ± 14.2	92.0 ± 13.1	0.7 (–1.1 - 2.5)	0.407	2.7 (2.0 - 3.9)	3.0 (2.3 - 4.5)	0.97	2.3	2.3
Mean#	86.7 ± 12.6	87.5 ± 13.1	0.7 (–0.2 - 1.6)	0.117	1.4 (1.0 - 2.0)	1.6 (1.2 - 2.4)	0.99	2.2	2.5
Rater 1 MRI									
Proximal	84.0 ± 14.7	82.4 ± 14.0	–1.6 (–4.7 - 1.6)	0.318	4.6 (3.5 - 6.9)	6.0 (4.5 - 8.9)	0.91	4.8	5.3
Mid	87.8 ± 14.9	88.7 ± 15.3	0.9 (–2.3 - 4.1)	0.560	4.6 (3.5 - 6.9)	5.8 (4.3 - 8.7)	0.92	2.8	2.9
Distal	91.1 ± 16.6	90.4 ± 13.8	–0.6 (–5.2 - 4.0)	0.778	6.7 (5.1 - 9.9)	7.6 (5.7 - 11.4)	0.82	1.9	1.9
Mean#	87.6 ± 14.3	87.2 ± 13.5	–0.4 (–2.7 - 1.9)	0.700	3.6 (2.5 - 5.0)	4.1 (3.1 - 6.1)	0.95	1.3	1.4
Rater 2 US									
Proximal	83.5 ± 14.3	84.9 ± 14.2	1.4 (0.2 - 2.5)	0.028*	1.8 (1.3 - 2.6)	2.1 (1.6 - 3.1)	0.99	4.2	4.6
Mid	89.3 ± 14.7	89.0 ± 14.2	–0.4 (–1.8 - 1.0)	0.574	2.0 (1.5 - 3.0)	2.3 (1.7 - 3.4)	0.98	1.2	1.2
Distal	91.1 ± 14.1	91.2 ± 13.6	0.1 (–1.9 - 2.2)	0.894	3.0 (2.3 - 4.4)	3.2 (2.4 - 4.8)	0.96	0.4	0.4
Mean#	88.0 ± 14.0	88.4 ± 13.5	0.4 (–0.5 - 1.3)	0.400	1.3 (1.0 - 2.0)	1.5 (1.1 - 2.2)	0.99	1.1	1.2
Rater 2 MRI									
Proximal	84.5 ± 14.0	83.1 ± 13.0	–1.3 (–4.8 - 2.1)	0.423	5.1 (3.8 - 7.5)	6.4 (4.8 - 9.6)	0.87	4.1	4.6
Mid	88.3 ± 16.2	88.3 ± 15.4	0.0 (–3.1 - 3.1)	0.997	4.5 (3.4 - 6.7)	5.5 (4.1 - 8.2)	0.93	0.0	0.0
Distal	90.8 ± 15.4	91.9 ± 13.9	1.1 (–3.5 - 5.7)	0.626	6.7 (5.1 - 9.9)	8.0 (6.0 - 12.0)	0.81	3.3	3.4
Mean#	87.8 ± 13.9	87.8 ± 13.1	–0.1 (–2.1 - 2.0)	0.931	3.0 (2.3 - 4.5)	3.7 (2.8 - 5.6)	0.96	0.3	0.3

Data are presented as mean ± SD. CI = confidence interval; *P* = paired sample t-test; TE = typical error; CV = coefficient of variation expressed as a percentage; ICC = intraclass correlation coefficient; SEM = standard error of measurement (mm^2); SEM% = standard error of measurement expressed as a percentage of the mean; US = ultrasound imaging; MRI = magnetic resonance imaging; * = significantly different between measure 1 and measure 2; # = mean score of the proximal, mid and distal scores.

Table 3-3 Between-day intra-rater reliability for estimates of patellar tendon cross-sectional area using US imaging

	Visit 1 (mm^2)	Visit 2 (mm^2)	Bias (95% CI)	*P*	TE (95% CI)	CV (95% CI)	ICC	SEM	SEM%
Rater 1									
Proximal	81.4 ± 12.5	83.3 ± 14.7	2.0 (–0.4 - 4.3)	0.096	3.4 (5.6 - 5.0)	4.1 (3.1 - 6.1)	0.94	6.0	6.9
Mid	87.5 ± 13.5	88.4 ± 14.2	0.9 (–1.5 - 3.4)	0.426	3.5 (2.7 - 5.2)	4.1 (3.1 - 6.2)	0.94	2.9	3.1
Distal	91.3 ± 14.2	90.9 ± 14.8	–0.4 (–2.5 - 1.8)	0.737	3.2 (2.4 - 4.7)	3.4 (2.6 - 5.1)	0.96	1.1	1.1
Mean#	86.7 ± 12.6	87.6 ± 13.9	0.9 (–0.7 - 2.4)	0.278	2.3 (1.8 - 3.5)	2.7 (2.0 - 4.0)	0.97	2.6	2.9
Rater 2									
Proximal	83.5 ± 14.3	84.1 ± 14.1	0.5 (–1.5 - 2.6)	0.599	3.0 (2.3 - 4.5)	3.8 (2.9 - 5.7)	0.96	1.6	1.8
Mid	89.3 ± 14.7	88.2 ± 14.3	–1.2 (–3.7 - 1.3)	0.335	3.7 (2.8 – 5.5)	4.2 (3.2 – 6.3)	1.00	3.7	4.2
Distal	91.1 ± 14.1	90.3 ± 14.5	–0.8 (–2.6 - 1.0)	0.339	2.6 (2.0 - 3.9)	3.0 (2.2 - 4.4)	0.97	2.6	2.7
Mean#	88.0 ± 14.0	87.5 ± 13.8	1.6 (1.2 - 2.3)	0.344	1.6 (1.2 - 2.3)	1.8 (1.4 – 2.7)	1.00	1.5	1.6

Data are presented as mean ± SD. CI = confidence interval; *P* = paired sample t-test; TE = typical error; CV = coefficient of variation expressed as a percentage; ICC = intraclass correlation coefficient; SEM = standard error of measurement (mm^2) ; SEM% = standard error of measurement expressed as a percentage of the mean; US = ultrasound imaging; * = significantly different between visit 1 and visit 2; # = mean score of the proximal, mid and distal scores

Table 3-4 Within-day inter-rater reliability for estimates of patellar tendon cross-sectional area using US and MRI

	Rater 1 (mm^2)	Rater 2 (mm^2)	Bias (95% CI)	*P*	TE (95% CI)	CV (95% CI)	ICC	SEM	SEM%
Ultrasound									
Proximal	81.4 ± 12.5	83.5 ± 14.3	2.2 (–0.4 - 4.7)	0.890	3.7 (2.8 - 5.5)	4.6 (3.4 - 6.8)	0.93	6.6	7.6
Mid	87.5 ± 13.5	89.3 ± 14.7	1.8 (–1.0 - 4.8)	0.203	4.3 (3.2 - 6.3)	4.8 (3.6 - 7.2)	0.92	5.7	6.0
Distal	91.3 ± 14.2	91.1 ± 14.1	–0.2 (–2.4 - 2.0)	0.858	3.3 (2.5 - 4.8)	3.9 (3.0 - 5.9)	0.95	0.6	0.6
Mean#	86.7 ± 12.6	88.0 ± 14.0	1.3 (–0.4 - 2.9)	0.127	2.4 (1.8 - 3.6)	3.0 (2.3 - 4.5)	0.97	3.9	4.3
MRI									
Proximal	84.0 ± 14.7	84.5 ± 14.0	0.5 (–1.0 - 2.0)	0.503	2.2 (1.7 - 3.3)	3.0 (2.3 - 4.5)	0.98	1.5	1.7
Mid	87.8 ± 14.9	88.3 ± 16.2	0.4 (–1.5 - 2.4)	0.649	2.8 (2.2 - 4.2)	3.2 (2.4 - 4.8)	0.97	1.3	1.4
Distal	91.1 ± 16.6	90.8 ± 15.4	–0.3 (–1.6 - 1.1)	0.691	2.0 (1.5 - 2.9)	2.1 (1.6 - 3.1)	0.99	0.8	0.8
Mean#	87.6 ± 14.3	87.8 ± 13.9	0.2 (–0.8 - 1.2)	0.653	1.5 (1.1 - 2.2)	1.6 (1.2 - 2.4)	0.99	0.7	0.8

Data are presented as mean ± SD. CI = confidence interval; *P* = paired sample t-test; TE = typical error; CV = coefficient of variation expressed as a percentage; ICC = intraclass correlation coefficient; SEM = standard error of measurement (mm^2); SEM% = standard error of measurement expressed as a percentage of the mean; US = ultrasound imaging; MRI = magnetic resonance imaging; # = mean score of the proximal, mid and distal scores

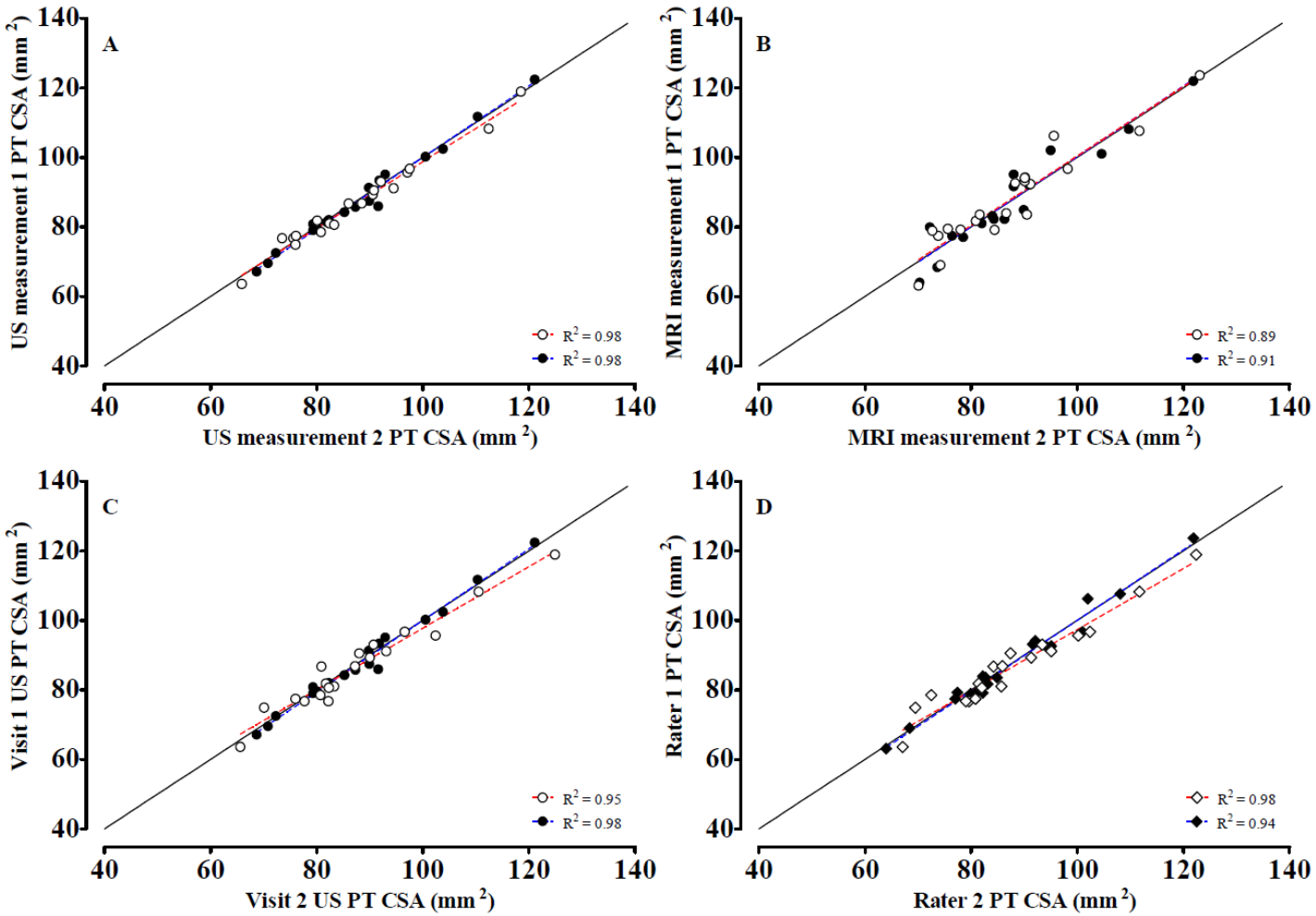

Figure 3-4. Panel A = Within-day, intra-rater reliability of estimates of PT CSA using US, Panel B = Within-day, intra-rater reliability of estimates of PT CSA using MRI, Panel C = Between-day, intra-rater reliability of estimates of PT CSA using US Panel D = Within-day inter-rater reliability of estimates of PT CSA using US and MRI. The solid black line represents the line of equality. In panels A, B and C, the dashed red line denotes the regression line for rater 1, and the dashed blue line denotes the regression line for rater 2. In panel D, the dashed red line denotes the regression line for US, and the dashed blue line denotes the regression line for MRI. The white dots represent individual data points for rater 1. The black dots represent individual data points for rater 2. The white diamond represents data points for US. The black diamond represents data points for MRI. PT = patella tendon; CSA = cross sectional area; US = ultrasound imaging; MRI = magnetic resonance imaging.

3-4 DISCUSSION

The aims of this study were to 1) determine the agreement between US and MRI measures of PT CSA for two independent raters 2) determine the within-day, inter- and intra-rater reliability for US and MRI measures of PT CSA and, 3) determine the between-day, intra-rater reliability of US measures of PT CSA. This study has demonstrated that there are high levels of agreement between US and MRI derived measures of PT CSA. Moreover, both US and MRI provide reliable within-day inter- and intra-rater measures of PT CSA. Finally, US provides reliable between-day, intra-rater measures of PT CSA. The results of this study are of particular importance as rater 1 will conduct measures of PT dimensions in the studies in chapters 4 and 5. As tendon dimensions will be used to calculate the mechanical properties of the tendon during exercise in the subsequent studies in this thesis, the high reliability of PT CSA measures by rater 1 in the current study can provide confidence that any changes discovered over time are unlikely to be due to variability in the measurement technique.

Agreement between MRI and US measures

Patellar tendon CSA values measured using US were systematically smaller compared with MRI for rater 1 at the proximal and mid regions of the PT (–2.6 mm^2, and –5.3 mm^2, respectively) but no systematic differences were found for the more experienced rater 2. In agreement with the present study, Stenroth et al. (2019) found that US derived measures of the AT were smaller than MRI measures, when the rater was inexperienced. However, for the PT, the more experienced rater underestimated CSA (mean of proximal mid and distal CSA) values derived from US measures in comparison to MRI, though the inexperienced rater showed no systematic difference between the two methods for the PT. In contrast, Kruse et al. (2017) reported consistent underestimation of AT CSA by US in comparison to MRI for both raters,

although their experience was not specified. Given the contrasting results, there appears to be no consistent link between rater experience and US vs MRI tendon CSA comparison.

Though there was systematic underreporting of proximal and mid PT CSA measures by US, compared to MRI in the present study, there were no systematic differences between US and MRI for either rater when all three sites (proximal, mid and distal) were combined and averaged for each participant. This is an important finding, as the mean score is commonly used to estimate average tendon CSA and subsequently calculate tendon stiffness and YM (Onambele et al., 2007, Murtagh et al., 2018, Maganaris and Paul, 1999, Kongsgaard et al., 2007, Hicks et al., 2013, Couppe et al., 2016, Couppe et al., 2008). Stenroth et al. (2019), who also used the method of measuring tendon CSA by averaging the proximal, mid and distal CSA values, reported that agreement between MRI and US measures of PT CSA was greater for the experienced rater (Pearson's $r = 0.87$), who had experience of musculoskeletal imaging (similar to the experienced rater in the current study) in comparison to the inexperienced rater (Pearson's $r = 0.44$). In comparison, the present study found similar agreement between the experienced (Pearson's $r = 0.44$). In comparison, the present study found similar agreement between the experienced ($r = 0.97$, SEE = 3.3 mm^2) and less experienced rater ($r = 0.98$, SEE = 2.6 mm^2), providing evidence that rater experience was not essential to levels of agreement. Additionally, agreement between MRI and US measures of PT CSA for both raters in the present study was higher than that for the experienced rater in the study by Stenroth et al. (2019). One potential reason for the difference between the studies could be due to the present study using a stronger magnet (0.35 T) in the MRI machine than the study by Stenroth et al. (2019) (0.18 T), with higher magnetic fields having better signal to noise ratios, typically producing higher quality images, though this does not necessarily result in more reliable images

(Trattnig et al., 1997). To date, no studies have directly assessed the effect of MRI magnet strength on estimating tendon dimensions, and the relationship between the two remain unclear.

Within-day intra-rater reliability

Rater 1 reported no systematic differences for within-day measures of US or MRI at any of the location specific measures or for combined PT CSA. Rater 2 overestimated PT CSA on measure two in comparison to measure one (1.4 mm^2) for US, whereas no systematic differences between measures for MRI were reported. The overestimation of PT CSA by US at the proximal site might be explained by two reasons. Firstly, in an attempt to sharpen the image quality, rater 2 might have re-positioned the probe distally, during the second measure. Although exploratory, this explanation is supported by a greater CSA reported at the mid-tendon, therefore any distal movement in US probe could explain the inflated estimation of PT CSA at the proximal site. The second reason might be attributed to a slight adjustment in probe orientation whilst scanning. Slight adjustments in probe angle can result in an increased diameter when positioned slightly askew (Gellhorn and Carlson, 2013), much like cutting into a cucumber at a bias. Nevertheless, the systematic difference in the present study was confined to the proximal site of the PT, with no difference occurring when the three locations were combined, which, as mentioned previously, is the measure used to further calculate Young's modulus (Murtagh et al., 2018, Kongsgaard et al., 2007, Hicks et al., 2013, Couppe et al., 2016, Couppe et al., 2008).

Within-day, intra-rater reliability of PT CSA estimations has received little study, with the available literature producing differing results. In the present study, relative reliability for US derived estimation of PT CSA, when proximal, mid and distal sites are averaged, was considered excellent, with an ICC of 0.99 for both raters which, in comparison to Gellhorn and

Carlson (2013) and Ekizos et al. (2013), who reported ICCs of 0.87-0.96 and 0.79, respectively, suggests a higher level of reliability, thus giving confidence that rater 1 can produce highly reliable measures of PT CSA, which will be used to assess Young's modulus in studies 2 and 3.

To the best of my knowledge the present study is the first to investigate the within-day, intra-rater reliability of MRI estimates of PT CSA, making comparison to other literature difficult. Two comparisons that can be made are from the studies by Kubo et al. (2001) and Stenroth et al. (2019) who assessed PT CSA estimations by MRI over two separate days. KUBO (2001) reported a CV of 1.6% where as Stenroth et al. (2019) reported CVs of 4.1% and 6.0% for experienced and inexperienced raters, respectively. The CVs of 4.1% and 3.7% in the present study for rater 1 and rater 2, respectively, are more in line with Stenroth et al. (2019) than Kubo et al. (2001). It is possible that the higher reliability displayed by Kubo et al. (2001) is due to the small sample size of six participants which can affect estimates of error (Springate, 2011), in comparison to 19 and 15 participants in the present study and the study by Stenroth et al. (2019), respectively. Moreover, it is unclear if the reliability figure presented by Kubo et al. (2001) is obtained from a single, experienced rater or not. If so, it would be possible that this is the reason for the high reliability presented, as it has been suggested that reliability can be optimised by using one experienced rater (Thoirs and Childs, 2018).

An unexpected finding in the present study was that for both raters, both relative reliability and absolute reliability was higher for US in comparison to MRI for all locations and mean PT CSA estimations, though reliability for all mean estimations for US and MRI PT CSA were considered excellent (ICC = $\geq$ 0.95). The most likely estimation for this is the technique used to determine the examination positions. The US examinations in the present study determined

the three measurement locations (proximal, mid and distal) prior to the scan being performed. In the MRI examinations, the image chosen for CSA analysis was chosen post-test, using the sagittal scan as a guide. Though anatomical landmarks were still used to determine measurement locations, slight variation in the positioning of the participant might have led to the images being analysed being slightly biased towards the proximal or distal insertion of the PT. Most likely, this was a combination of both, which would explain the lower reliability but a lack of a systematic difference between measures.

Between-day intra-rater reliability

Due to MRI availability, between day reliability, assessed by two visits, three days apart, was assessed for US measures only. In comparison to Stenroth et al. (2019), who also estimated PT CSA on two separate occasions using US, the present study showed higher reliability. Relative reliability was higher for both raters within the present study (ICCs 0.97 and 0.99, rater 1 and rater 2, respectively) in comparison to both raters (ICCs 0.85 and 0.50, respectively) in the study by Stenroth et al. (2019), with their second rater being less experienced than the first. Raters 1 and 2 in the present study also displayed higher absolute reliability in comparison to the two raters in the study by Stenroth et al. (2019), with SEMs of 2.6 and 1.5 mm^2 vs 5.0 and 8.9 mm^2, respectively. Consistent with the lower reliability reported for within-day, intra-rater US measures of PT CSA, Ekizos et al. (2013) reported lower reliability (mean ICC 0.60) than the present study and Stenroth et al. (2019). Ekizos et al. (2013) attributed the low levels of reliability to low image quality obtained from US, making the distinction of the tendon borders difficult. This appears to not be the case both in the present study and the reliability reported by Stenroth et al. (2019). One explanation for this could be the US machine used by Ekizos et al. (2013), as research has suggested that differences in equipment can affect intra-rater

reliability (Gellhorn and Carlson, 2013). Although, Ekizos et al. (2013) and the current study both used 7.5 Hz linear array probes, therefore and differences in reliability due to the equipment might be due to differences in manufacturer specifications, though this needs further investigation. Additionally, the technique used to define PT measurement sites by Ekizos et al. (2013) was to use the first image below the patellar bone and above the tibia as the location for the proximal and distal measurement, respectively. This therefore might open some subjectivity between raters as to where the proximal and distal regions are located, which could have differed between visits via slightly different probe orientation, for example. In the present study, and the study by Stenroth et al. (2019), anatomical landmarks (apex of the patellar and the tibia tuberosity) were used to define the origin and insertion of the PT, the proximal, mid and distal examination sites were then mathematically calculated based on these measurements. As this process was repeated on both visits, it would appear that this process might account for the higher reliability recorded in the present study and the study by Stenroth et al. (2019), in comparison to Ekizos et al. (2013), hence validating the importance of a consistent examination protocol which in turn, might improve reliability (Thoirs and Childs, 2018).

Inter-rater reliability

For both US and MRI, intra-rater reliability was considered excellent (ICC = ≥ 0.92), with MRI slightly higher in terms of relative and absolute reliability and no systematic differences present for any measures. Inter-rater reliability was considerably higher in the present study compared to Stenroth et al. (2019) for both relative (US ICCs 0.97 vs 0.56, MRI ICCs 0.99 vs 0.62) and absolute (US SEM 0.7 mm^2 vs 6.0 mm^2) reliability. The large inter-rater differences between the two studies can be attributed to the large difference in experience of the raters in the study by Stenroth et al. (2019), with the inexperienced rater having no prior experience in

musculoskeletal radiography. In the present study, although rater 1 was less experienced than rater 2, a substantial level of practice with the digitisation process was undertaken before the study. Research has shown that increased experience can improve the reliability of US measures (Dudley-Javoroski et al., 2010) although it remains to be determined exactly what level of experience is needed to produce high levels of reliability.

Limitations

The present study provides important methodological evidence which will allow the interchangeable use of US and MRI in estimating PT CSA. However, this study is not without its limitations. Specifically, the estimation of PT CSA for both US and MRI were based on the judgements of the raters and their interpretation of the tendon borders. Although agreement between the two studies was excellent, it cannot be ruled out that the true CSA is what was measured by MRI analysis. It is difficult to ascertain if both the US and MRI images included the paratenon, due to it not being clearly identifiable (Bohm et al., 2016). This gross over- or under-estimation might have consequences for subsequent mechanical calculations pertaining to PT CSA (e.g. Young's Modulus), and whilst within study comparisons would not be affected, extrapolation to other populations might be difficult. In addition, despite the procedures within the study being of high consistency, they do differ to other PT CSA estimation studies (e.g. examination location).

Another limitation is the time period between the test days of the US measurements. With only three days in between each measure, the test-retest reliability of scores over longer time periods, is not known. Whilst this approach ensures that the US measures are comparable, it does not consider the potential change in diameter of tendons that can occur over time with exercise (Tardioli et al., 2012). With the subsequent chapters in this thesis including an

exercising population, and studying responses over longer time periods (i.e. the RBE), these physiological considerations must be considered. Finally, caution must be taken if future research utilises equipment that is different to that used in the present study or uses raters of different musculoskeletal radiography experience, as this might affect the reliability of any subsequent results.

Conclusion

This present study shows that PT CSA obtained via the use of US is a reliable tool both within and between operators. Moreover, US and MRI can be used interchangeably to assess PT CSA; this would not be recommended within the same study but could be used as a way of comparing studies with different means of image acquisition. Although this study contradicts reports of reliability from earlier research, this is most likely dependent on the strictness of scanning protocol and operator experience. In subsequent chapters in this thesis, rater 1 will be performing all US scanning procedures. Therefore, it is with confidence that the PT CSA measures obtained by the rater will be accurate and reliable. As such, the subsequent chapter will measure tendon forces during exercise, with the PT CSA measure being used to calculate YM. In the wider context of the thesis, this measure of YM will be used to determine changes in the behaviour of the PT following maximal ECC exercise, with this change in tendon behaviour proposed to be a mechanistic underpinning of the RBE (Lau et al., 2015, Hyldahl et al., 2017).

CHAPTER 4 TEST-RETEST RELIABILITY OF MEASURING PATELLA TENDON PROPERTIES AND MUSCLE FASCICLE LENGTH CHANGES IN THE VASTUS LATERALIS DURING MAXIMAL ECCENTRIC EXERCISE, USING 2D B-MODE ULTRASONOGRAPHY

4-1 INTRODUCTION

Chapter 3 confirmed that US is a valid and reliable method of measuring PT CSA which is important due to PT CSA being part of the equation used to calculate YM (Onambele et al., 2007). However, PT CSA is a resting measure and can only partially account for the behavioural properties of tendon during dynamic exercise. During maximal isometric knee extension exercise, PT properties such as stiffness and YM can also be measured using US (Onambele et al., 2007, Hicks et al., 2013, Fukunaga et al., 1996). Moreover, US can also be used to measure muscle behaviour during maximal knee extension exercise (Penailillo et al., 2015, Hicks et al., 2017, Hicks et al., 2013, Guilhem et al., 2016). The ability to measure both tendon and muscle behaviour in real time during exercise makes US an attractive prospect for researchers, given that there is evidence to suggest that the behaviour of the muscle is mediated by properties of the tendon (Hicks et al., 2013, Guilhem et al., 2016).

Magnetic resonance imaging is an alternative method of measuring muscle and tendon properties and has some advantages over US, such as the ability to measure large areas of muscle simultaneously (Lieber and Ward, 2011, Finni, 2006) and has high spatial resolution, leading to high image quality (Lieber and Ward, 2011). However, MRI is expensive and unlike US, usually requires participants to remain stationary (Narici, 1999), limiting the dynamic measurement of muscle and tendon properties. This typically leads to researchers using US to measure muscle and tendon behaviour when movement is involved. An immediate advantage of the use of US in dynamic movements is that US allows the quantification of the independent contribution of muscle and tendon to total MTU behaviour (Reeves and Narici, 2003, Fukunaga et al., 1997), which is important so that the relationship between muscle and tendon properties and their behaviour during movement can be investigated.

Many variables can be quantified by US during dynamic activity; however, muscle fascicle behaviour has received attention in the literature for a number of reasons. Firstly, muscle fascicles are closely related to sarcomere length (Lichtwark et al., 2018), which has been shown to have an indirect relationship with muscle energetics and force production (Bohm et al., 2018). Moreover, during ECC exercise, the magnitude of muscle fascicle lengthening has been shown to influence the muscle damage response (Guilhem et al., 2016, Hicks et al., 2017). Additionally, reduced muscle fascicle lengthening during the second of two identical bouts of ECC exercise has been associated with a reduced magnitude of EIMD (Penailillo et al., 2015). There is also evidence that muscle fascicle lengthening during maximal ECC exercise is greater for males in comparison to eumenorrheic females, and that this leads to a higher amount of circulating CK levels in the blood (Hicks et al., 2016), though no fascicle lengthening differences have been shown between eumenorrheic females and OCP using females. Nevertheless, given the evidence that the degree of fascicle lengthening can be related to EIMD magnitude, it is important that fascicle lengthening is measured as accurately and reliably as possible to confirm this relationship.

The reliability of any measurement tool must be established before there can be confidence in any result obtained from the measure, with US being no exception. A systematic review by Kwah et al. (2013) assessed the use of US to measure muscle fascicle length and pennation angle (PA) and concluded that US generally provides valid and reliable measures. However, the majority of studies in this review were based on resting, passive or isometric conditions, making it difficult to extrapolate those findings to studies that investigate dynamic actions; specifically, maximal ECC exercise. Unlike isometric contractions, there is substantial lengthening of the MTU during ECC contractions, which can be altered by lengthening of the tendon (Lichtwark and Wilson, 2007, Joseph et al., 2016), the muscle fascicles (Hicks et al.,

2013, (Penailillo et al., 2015), or a combination of both (Guilhem et al., 2016). Additionally, it has been reported that MTU behaviour is different between males and females (Hicks et al., 2013). More recently, Van Hooren et al. (2020) concluded that US was a reliable method of determining fascicle length and PA during movement, including ECC exercise, if certain recommendations were incorporated. These included using probe holders to minimise alterations in probe orientation, using probes with a large field of view, and to maximize sampling frequency by minimising image depth. Therefore, as reliability is potentially dependent on one or more of these factors, further research implementing these recommendations by Van Hooren et al. (2020) is warranted.

Obtaining US measurements of fascicle behaviour during lengthening actions is challenging, and it is essential to ensure high test-retest reliability. The systematic review by Van Hooren et al. (2020) included only one study that investigated the reliability of measuring VL fascicle lengthening during maximal ECC exercise. This study by Guilhem et al., (2011) reported ICCs of 0.82, 0.75 for VL fascicle lengthening and pennation angle, respectively. Additionally, this study was conducted on just five participants, over two visits separated by one day. In a subpopulation of five males, Hicks et al. (2013) investigated the reliability of VL fascicle lengthening during maximal ECC exercise across two visits, separated by four days, where an ICC of 0.998 was reported. These two studies reported excellent reliability [ICC $\geq$ 0.9 (Koo and Li, 2016)] but the short test-retest period does not account for any fluctuations in muscle fascicle architecture that might happen over time. In this thesis, the final study will be measuring PT and VL fascicle properties using US with ~ four weeks in between visits. Therefore, reliability of these US measures of PT and VL properties needs to be assessed with a four-week test-retest period, to give confidence that any findings in the final study between visits are due to the exercise intervention and not measurement error. Therefore, the aim of this

study was to determine the reliability of using US to quantify PT properties at rest and during maximal isometric exercise, and VL properties at rest and during maximal ECC exercise. This is to be completed with a large participant number, over three visits, which has been suggested as a minimum requirement for reliability studies (Hopkins, 2000). Moreover, no reliability studies investigating VL properties during maximal ECC exercise and tendon properties during maximal ISO exercise have included a female population, despite reported sex differences in patella tendon (Onambele et al., 2007) and VL fascicle lengthening (Hicks et al., 2013) during maximal exercise. Therefore, a subgroup analysis will be performed to determine the reliability of the same measures for males, females using the oral contraceptive pill and eumenorrheic females to reflect the participant groups to be used in the final study.

4-2 METHODS

4-2.1 Participants

A total of 36 participants were recruited for the study, and divided into three groups; 1) twelve males (age: 23 ± 4 years; stature: 1.79 ± 0.11 m; mass: 87.1 ± 10.8 kg); 2) 12 females (age: 22 ± 6 years; stature: 1.68 ± 0.08 m; mass: 62.3 ± 11.7 kg) taking a monophasic OCP; and 3) 12 eumenorrheic females (age: 24 ± 4 years; stature: 1.69 ± 0.06 m; mass: 69.1 ± 10.4 kg) who had not used any form of hormonal contraception for the past 12 months (self-reported). Participants completed a pre-test questionnaire (Appendix 2) and were included in the study only if they had no neuromuscular or musculoskeletal impairments in the lower limbs within the last six months. Female participants completed an additional menstrual health questionnaire (Appendix 2). All participants self-reported as being physically active, participating in at least one lower body resistance session per week. Females in the OCP group reported taking a combined monophasic OCP, with estradiol doses between 20-30 µg, for an average of 4 ± 2

years. The types of monophasic OCP used in this study were taken daily for 21 days, followed by a 7-day, withdrawal phase. Females in the eumenorrheic group reported a regular menstrual cycle (mean length 27 ± 2 days). A regular menstrual cycle was defined as 24-36 days (Landgren et al., 1980, Cole et al., 2009). Female participants with menstrual cycles outside of this regular range, or females who were pregnant in the year preceding the study were excluded. Institutional ethical approval was received from the Northumbria University Faculty of Health & Life Sciences Ethics committee in accordance with the *Declaration of Helsinki*. Participants were supplied with a participant information sheet, detailing the purpose of the study and gave written consent before participating (Appendix 1).

4-2.2 Experimental design

Participants were asked to visit the laboratory on four occasions; one practice session and three experimental visits. The practice visit included measures of anthropometrics, PTMA and dynamometer familiarisation. The three experimental trials consisted of resting measures of VL architecture and PT dimensions using US. Additionally, measures of PT stiffness and optimal angle of force production of force production (OA) during iMVCs were recorded. Following that, VL fascicle lengthening was recorded using US during maximal ECC knee extension exercise. The first and second experimental visit were separated by one week. The first and third experimental visits were separated by four weeks for males and OCP taking females. For eumenorrheic females, to ensure consistency in the menstrual cycle phase between two consecutive cycles, and to reflect the menstrual cycle phase in which the eumenorrheic females in chapter 5 were to be tested, the first and third experimental visits were during the mid-luteal phase of the menstrual cycle (~four weeks apart), calculated from a positive ovulation test, and retrospectively confirmed by serum hormone analysis.

Practice session

The practice session began with the collection of stature and mass, measured using a portable stadiometer (Seca model 213; Seca, Hamburg, Germany) and digital scales (Seca model 813; Seca, Hamburg, Germany) respectively. This was followed by a dual-energy X-ray absorptiometry scan (DXA) to measure the PTMA of the non- dominant leg (defined as the leg used to provide stability during movements e.g. kicking) at a 90° knee angle (0° = full extension). The participant was then familiarised with the physical requirements of the testing procedure. This involved sitting in an isokinetic dynamometer (System 4 Pro, Biodex Medical Systems Inc., NY, USA) and taking the non-dominant leg passively through a pre-determined range of motion (30°-110° knee extension). Following three sub-maximal efforts, participants then performed iMVCs of the knee extensors against the lever arm of the dynamometer at 70° and 80° knee angles to practice contractions at different knee angles. This was followed by a ramped iMVC at 80° knee angle, with the aim of reaching their previous iMVC torque in a time of 4-6 seconds, as linear as possible, as this was the contraction type used to assess tendon stiffness (see below). The knee angle used in the practice session was reduced by 10° in comparison to the experimental visits as iMVCs at long muscle lengths can confer a protective effect against EIMD in subsequent exercise sessions (Chen et al., 2012b). Participants received a demonstration of maximal ECC contractions of the knee extensors to familiarise the participant with the action of the dynamometer. No ECC knee extensor exercise was included in the practice trial, as previous research has demonstrated that even very small volumes of ECC exercise can confer a protective effect against EIMD on subsequent bouts (Nosaka, 2001).

Experimental visit

Visit 1 began with the collection of resting measures of VL length, width, thickness, CSA, fascicle length (FL), and PA using US, in a supine position to control for changes in intramuscular fluid between lying and standing and to enhance US image quality (Berg et al., 1993). Participants then transferred to the dynamometer where they were seated in an upright position and the non-dominant leg positioned at 90°. Patella tendon length and CSA were then recorded (described in section 3-2.2). Following three sub-maximal warm up attempts, participants then performed two ramped iMVCs whilst patella and tibia tuberosity displacement was measured to assess tendon elongation, which was subsequently used to calculate tendon stiffness (described below). Following this, the OA of force production was determined by a series of iMVC contractions at different knee angles (described below). Following this, two sets of five maximal ECC contractions of the knee extensors, through a range of motion between 30°- 110° knee angle (0° = full extension), were performed in the isokinetic dynamometer at an angular velocity of $30°\cdot s^{-1}$ during the ECC phase, and an automatic passive return to full extension at $60°\cdot s^{-1}$ in the CON phase, with 120 s between sets.

Visits 2 and 3 followed the same protocol as visit 1, one week and four weeks later, respectively, and data obtained were assessed for reliability. One -week retest reliability (visit 1 to 2) was used to assess the reliability of all ultrasound-based measures with minimal biological variability. Four-week retest reliability (visit 1 to 3) was assessed as the variability across this time period was considered important as it replicated the proposed intervention duration of the subsequent experimental chapter (chapter five) of this thesis. In between visits, participants were instructed to live habitually, with the exception of avoiding lower body exercise for 24 h prior to each visit.

For the initial experimental visit, females taking the OCP were tested on the day of the 14th pill ingestion (self-reported). Eumenorrheic females were tested during the mid-luteal phase of the menstrual cycle. This was achieved by confirming ovulation and testing these participants approximately seven days (± one day) after. This procedure was implemented before experimental visits one and three for this population. For eumenorrheic females, experimental visit one will take place in the mid-luteal phase of the menstrual cycle and visit two, that will assess the seven-day reliability, will take place in the late luteal or early follicular phase of the menstrual cycle. Although visits one and two will be in slightly different stages of the menstrual cycle and therefore might have differences in circulating levels of oestrogen and progesterone, the strength of evidence suggests that short term fluctuations in these hormones across the menstrual cycle does not influence the mechanical properties of human muscle and tendon (Kubo et al., 2009). A detailed description of all experimental procedures can be found below.

4-2.3 Procedures

Anthropometric measurements

Stature and mass were measured as described in section 3-2.3. Mid-thigh circumference (MTC) was defined as the mid-point between the *Trochanterion* and *Tibiale laterale* and measured using a standard anthropometric tape.

Vastus Lateralis fascicle length and pennation angle

A real-time B-mode ultrasound (HDI 5000 SonoCT, Philips, Amsterdam, Netherlands) was used to obtain resting measures of VL FL, and CSA of the non-dominant leg. With the participant laying in a supine position with the leg fully extended, an ultrasound probe (7.5 MHz linear array probe, 50 mm wide) was used to identify the distal and proximal insertion

sites of the VL, where marks were placed on the skin, with the distance between the two marks recorded as VL length. At 50% VL muscle length, the border where the VL meets the *rectus femoris* (RF) and *biceps femoris* (BF) was located using the ultrasound probe and marked on the skin. The distance between the VL-RF and VL-BF boundaries indicated VL muscle width. The location where 50% muscle length and width met was denoted by a cross in permanent marker. This was the location of the ultrasound probe during all measures of VL muscle fascicle length. Additionally, this process of determining the location of the ultrasound probe was repeated for each individual visit. An echo absorptive marker was placed on the skin at the measurement location to provide a visual baseline of the internal VL structure and to ensure that there was no movement artefact confounding the measurement of FL. Resting VL FL was taken at this location by placing the ultrasound probe perpendicular to the skin in the mid-sagittal plane and orienting the probe as precisely as possible with the fascicle. Ultrasound images were recorded in real time at 25 frames per second using image acquisition software (AVer Media Capture Studio, AVer Media Technologies, New Taipei City, Taiwan) and analysed offline using imaging software (ImageJ 1.45; National Institutes of Health, Bethesda, MD, USA). Fascicle length was measured from the visible insertion of the fibre into the deep or superficial aponeurosis. If the fascicle extended beyond the width of the ultrasound image, linear continuation to calculate FL was applied (Figure 4-1), with this method of measuring FL typically yielding an error of 2-7% (Finni et al., 2003, Finni et al., 2001). To reduce error using the linear continuation method, an average of three fascicles was assessed for each image. In each image where VL FL was determined, PA for the corresponding fascicle was measured (Figure 4-1). The PA was determined as the angle between the deep aponeurosis and the muscle fascicle (Kubo et al., 2001).

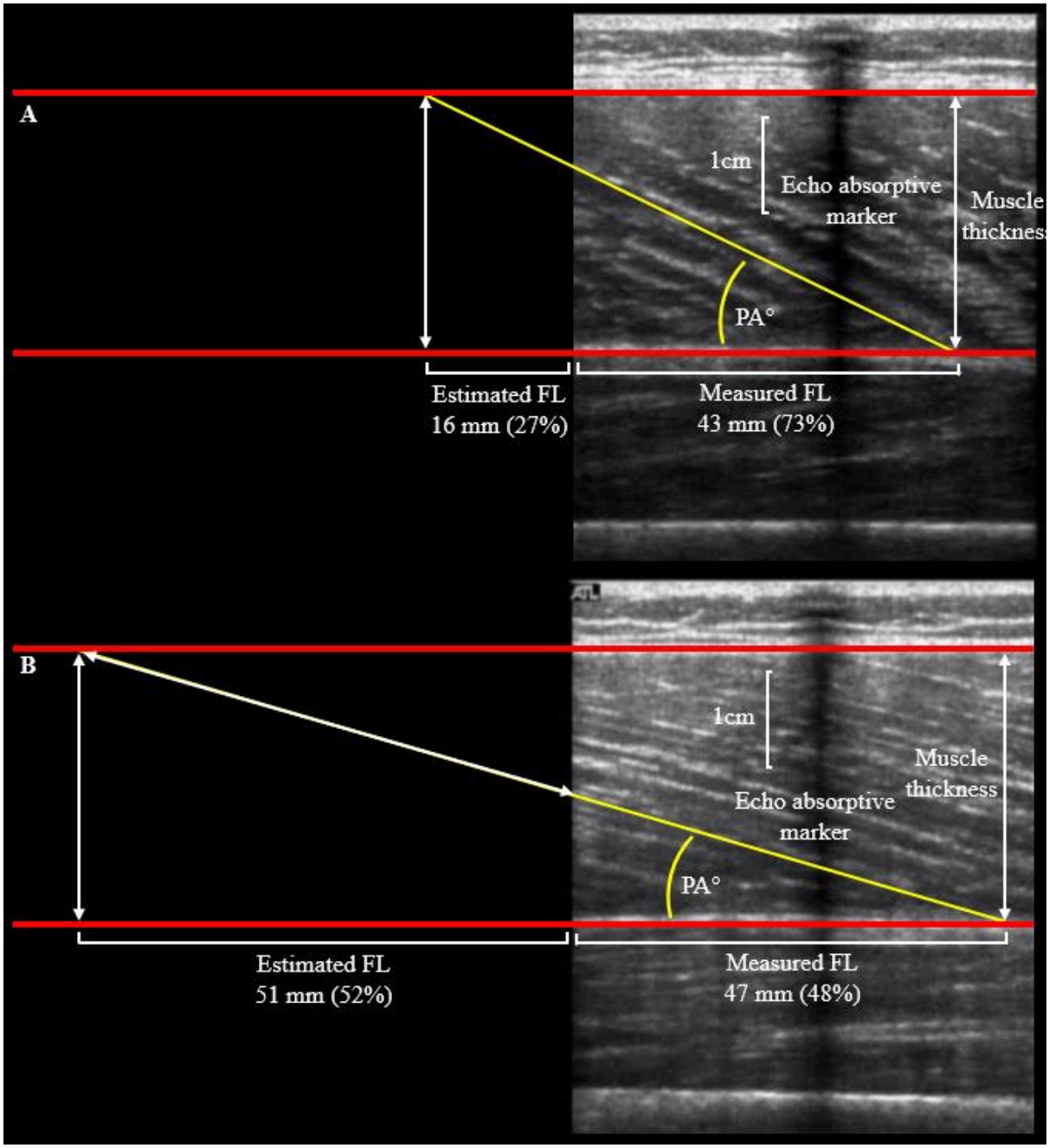

Figure 4-1 An example of the linear continuation method used to estimate VL fascicle length at rest and measures of muscle thickness and pennation angle (PA). A) 30° knee angle (0° = full extension) B) 110° knee angle.

Vastus lateralis anatomical cross-sectional area and muscle thickness

To measure anatomical VL CSA, at 50% VL length, echo-absorptive markers were placed at 30 mm intervals from the medial to the lateral edge of the VL muscle. The ultrasound probe was placed in the axial plane, perpendicular to the VL muscle and moved slowly across the echo-absorptive markers. The images obtained were aligned using the shadows cast by the echo-absorptive markers and the contour of the muscle. This allowed the entire VL cross-sectional area to be re-created using imaging software (Adobe Photoshop Elements, version 15, Adobe). The VL cross-sectional area was then measured using the imaging software described previously.

Vastus lateralis muscle thickness was measured by placing the US probe in the transverse plane perpendicular to the skin. The site of measurement was the same as where VL FL was measured, at 50% VL length and width. Thickness of the VL was measured as the distance between the adipose tissue-VL interface and the VL-*vastus intermedius* interface (Kubo et al., 2001) which is displayed in Figure 4-1.

Tendon length and cross-sectional area

Patella tendon length and CSA were assessed using the methods described in section 3-2.3.

Patella tendon stiffness

To measure PT stiffness, participants performed isometric actions in an isokinetic dynamometer (System 4 Pro, Biodex Medical Systems Inc., NY, USA). The dynamometer settings and 0° knee extension (full extension) used during the initial experimental visit were recorded and standardised throughout all subsequent visits. Prior to performing any actions,

the knee was fixed by inextensible straps at 90° and inextensible straps were placed over the hips and shoulders to minimise any extraneous movement.

The participant was instructed to perform a ramped iMVC lasting approximately six seconds in the isokinetic dynamometer. Patella tendon displacement was assessed in the sagittal plane via ultrasound at both the apex of the patella and the tibial tuberosity. This was recorded over two contractions, separated by 90 s, consistent with Onambele et al. (2007) as a means of measuring PT displacement. The combined displacement of the two sites was taken as total tendon displacement. Images were captured at approximately 10% increments of the ramped iMVC, synchronised with the torque acquisition system (Spike 2, Cambridge Electronic Design, Cambridge, UK) by an external square wave pulse. Patella tendon stiffness was calculated at every 10% of iMVC torque using the following formula: iMVC torque + antagonist co-activation torque / PTMA (see below).

Patella tendon moment arm of the non- dominant leg was measured at 90° using a DXA (Horizon™ DXA System, Hologic Inc, MA, USA), scan (IVAHD Image type), in order to calculate tendon forces. A goniometer was used to ensure a knee angle of 90°. Patella tendon moment arm was determined as the perpendicular distance between the centre point of the patella tendon and the tibio-femoral contact point (Figure 4-2) quantified using digitising software (MIPAV v8, NIH, MD, USA). This method of measuring PTMA has been shown to be valid and reliable when compared to MRI (Erskine, 2014).

The force × elongation curve derived from data at every 10% of maximal ramped iMVC was fitted with a second order polynomial function forced through zero (Onambele et al., 2007). The tangential slope at discreet sections of the curve, relative to maximal ramped iMVC force, was calculated by differentiating the curve at every 10% PT force interval. To account for

force differences between visits, the slope of the tangential line was computed for the weakest iMVC across the three visits. This standardised the comparison of PT stiffness and YM at a consistent load within each participant.

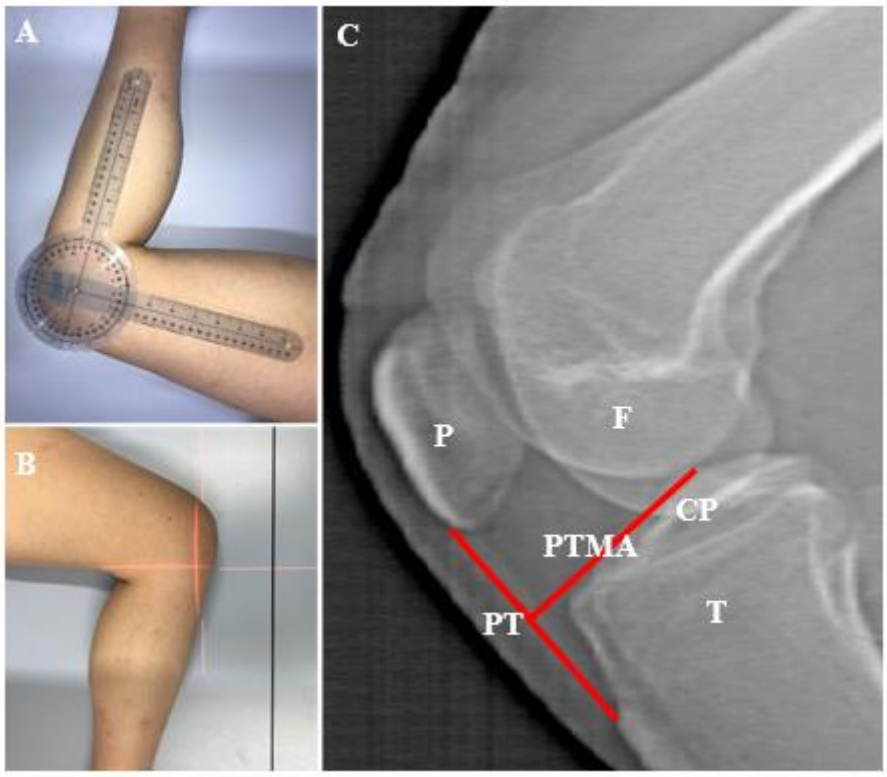

Figure 4-2 A) a participant positioned in the DXA scanner with the knee positioned at 90° B) scanning location identified using a target C) an example image of a DXA scan used to assess patella tendon moment arm. P = patella, F = femur, PT = patella tendon, PTMA = patella tendon moment arm, T = tibia, CP = tibio-femoral contact point

Antagonist co-activation

Surface electromyography EMG activity was recorded from the BF, *semitendinosus* (ST) and *semimembranosus* (SM) muscles during all contractions. To minimise interference and to ensure that electrode placement was over the belly of each muscle, US was used to define the medial and lateral borders of each muscle and marked with permanent marker. Electrode

placement was halfway between each marked site for each muscle. Site areas were shaved and cleaned prior to the placement of electrodes to reduce skin impedance below 5000 Ω (Hermens et al., 2000). Surface EMG bipolar electrodes (Delsys Trigno, Delsys, Boston, MA, USA) were placed on the muscle bellies and root-mean-square (RMS) amplitude was recorded during all contractions. EMG data was collected (2000 Hz), filtered (20-450 Hz), and acquired for off-line analysis (Spike 2, Cambridge Electronic Design, Cambridge, UK). Electrodes measuring EMG activity in the BF, SM and ST were surrounded by a protective sheath to minimise any potential interference caused by compression against the electrode from the seat of the dynamometer.

To determine antagonist co-activation during the ramped iMVC exercise, maximum EMG of the BF (long head), ST and SM during knee flexion iMVC was measured. Following three sub-maximal warm up attempts, participants performed two iMVC knee flexions at a 90° knee angle against the lever arm of the dynamometer, producing as much force as fast as possible, holding for 3-5 s before relaxing. The integral of the RMS was calculated, 500 ms either side of peak torque. During the ramped iMVCs of the knee extensors, at every 10% of peak torque, the absolute integral of the BF, ST and SM EMG were taken over 250 ms. The mean EMG of the knee flexor muscles was used to calculate co-activation torque. Co-activation torque was calculated as (KF EMG during ramped maximal voluntary isometric knee extension / knee flexor EMG during maximal voluntary isometric knee flexion) × peak maximal voluntary isometric knee flexion torque at 90° knee angle (Onambele et al., 2007). This equation assumes that a linear relationship exists between knee flexor EMG and iMVC knee flexion torque and is commonly used within the literature (Lippold, 1952).

Young's modulus

Young's modulus was calculated using the formula; PT stiffness × (PT length (mm) / PT CSA (mm^2) at ramped iMVC (Onambele et al., 2007). Young's modulus was standardised to the weakest iMVC across the three visits as previously explained in the patella tendon stiffness section.

Measures of maximal isometric force and optimal angle

After performing the isometric contractions required to calculate tendon stiffness and YM, participants then performed two iMVCs at seven different knee angles (60, 65, 70, 75, 80, 85 and 90°) in a randomised order, with 60 s between repetitions. Participants were asked to push against the lever arm of the dynamometer as fast and as forcefully as possible, holding the contraction for 3-5 s. The highest torque produced at each knee angle was taken as maximal isometric voluntary contraction. The angle at which the highest torque was produced was recorded as the optimum angle for force production.

Measurements of VL fascicle length during eccentric exercise

To measure VL fascicle behaviour during ECC knee extensions, the ultrasound probe was placed at 50% of VL muscle length and width (as previously defined), in the mid-sagittal plane of the non-dominant leg and fixed in position using a customised bracket (Figure 4-3). During ECC contractions from 30 to 110° knee angle, ultrasound images were recorded in real time at 25 frames per second using image acquisition software (AVer Media Capture Studio, AVer Media Technologies, New Taipei City, Taiwan). An externally generated square wave pulse was used to synchronise the images with the torque acquisition system that simultaneously recorded knee joint angle. An echo-absorptive marker was placed on the skin to ensure that

any probe movements during contraction could be identified during analysis (Figure 4-1). Images were analysed offline (at every 10° during eccentric contractions) using imaging software (ImageJ 1.45; National Institutes of Health, Bethesda, MD, USA). Fascicle lengths from the still images were analysed as previously described (Figure 4-2). Fascicle length changes were measured as absolute change (FL at 110° knee angle – FL at 30° knee angle) and relative change (percentage increase in FL from 30° (0%) knee angle to 110° (100%) knee angle). Three ECC contractions were analysed per visit. These were the fifth repetition of the first set and the second and fifth repetition of the second set. This allowed comparisons across visits of each individual repetition in turn (e.g. set one repetition five, compared across all three visits). Additionally, to measure within set reliability of VL fascicle lengthening, the second and fifth repetitions of the second set were compared. To measure the between-set reliability of VL fascicle lengthening, the fifth repetition of set one and set two were compared. For both within- and between-set reliability, the mean of all three visits per respective repetition was used for analysis.

Figure 4-3 An example of probe placement during ECC exercise using a custom-made bracket.

Confirmation of ovulation in eumenorrheic females

Prior to any testing, eumenorrheic females were required to confirm they were within the mid-luteal phase (~day 21) of the menstrual cycle. To achieve this, prior to visits 2 and 4, participants determined ovulation via the use of a home ovulation kit that detected surges in luteinising hormone (LH) (Clearblue digital, Swiss Precision Diagnostics Gmbh, Geneva, Switzerland). Participants were required to test their urine from day nine of the menstrual cycle, with day one being the first day of bleed. A surge of LH on two consecutive days confirmed that eumenorrheic females were ovulatory (Su et al., 2017). Following the confirmation of

ovulation, participants visited the laboratory seven days later, to ensure they were in the mid-luteal phase of the menstrual cycle (Mihm et al., 2011). On the day of experimental visits 2 and 4, prior to any of the experimental procedures described above, a 10 mL venous blood sample was collected from the antecubital fossa region of the arm (BD Vacutainer, Beckton Dickenson, Oxford, UK), to measure serum concentrations of estradiol and progesterone. For the samples to be analysed for estradiol, the collected blood was allowed to clot at room temperature for 30 minutes before being centrifuged at 1000 × g at 5 ˚C for 15 minutes. For the samples to be analysed for progesterone, the collected blood was allowed to clot at room temperature for two hours before being centrifuged at 1000 × g at 5 ˚C for 15 minutes. 50 µL of serum was pipetted into four separate aliquots and analysed for concentrations of estradiol and progesterone using commercially available ELISA kits (ELISA; Bio-techne, MN, USA), following manufacturer's instructions.

Data analysis

All ultrasound videos taken were exported to video editing software (Adobe Premier Elements version 15, Adobe, CA, USA) for frame-by-frame analysis. For resting measures of VL and PT, the required images were selected by the researchers for further analysis. For measures of VL and PT during exercise, the images that corresponded to the specific time point (synchronised with the data acquisition software) were selected for further analysis. All images were analysed using the imageJ software.

4-2.3 Statistical analysis

Data are presented as mean ± SD. The level of significance was set to $\alpha = 0.05$. Statistical analysis was performed using the statistical software package SPSS (v.19; Chicago, IL, USA) and using a published spreadsheet (Hopkins, 2015) in Microsoft Excel (Microsoft Excel 2016,

Microsoft, Washington D.C., USA). One-way repeated measures ANOVA were employed to assess for differences between visits, with the exception of within- and between-set VL fascicle lengthening, where a paired sample t-test was used to test for differences. Where a difference was found following the one-way repeated measures ANOVA, least significant post *hoc* tests were performed. Typical error (raw units), typical error as a coefficient of variation (CV, %), and intra-class correlations were calculated for all variables for the assessment of random error. Reliability via ICC was interpreted by the following: ICC 0.5 – 0.75, moderately reliable, ICC 0.75-0.9, good reliability, ICC > 0.9, excellent reliability (Koo and Li, 2016). Statistical significance was accepted at $P < 0.05$. Analyses were performed for all participants combined (n = 36) and within each experimental group (n = 12 for each group).

4-4 RESULTS

Vastus lateralis properties

Mean ± SD VL properties for all participants are presented in Table 4-1. Individual data points for VL architecture measures in visit 1 vs visit 2 and visit 1 vs visit 3 are displayed in Figure 4-4. CVs were 2.3%, 2.6%, 2.5% and 2.3% for VL CSA, FL PA and thickness, respectively. All measures of VL properties showed excellent reliability (ICC ≥ 0.97). Results from the ANOVA revealed that resting measures of VL FL and PA were different between visits. Post *hoc* analysis showed that US overestimated VL FL by 0.4 ± 0.9 mm in visit 1 in comparison to visit 3 ($p = 0.034$) and VL PA was 0.1 ± 0.3° greater in visit 1 in comparison to visit 3 ($p = 0.042$). No other systematic differences were present. Optimal angle of force production produced a CV of 7.7% and moderate reliability (ICC 0.57).

Patella tendon properties

Mean ± SD PT properties are presented in Table 4-2. Individual data points for PT property measures in visit 1 vs visit 2 and visit 1 vs visit 3 are displayed in Figure 4-5. CVs ranged from 1.0% to 11.5% for all measures. PT CSA, PT length, knee extension MVC, knee extension antagonist co-activation, PT force, PT stiffness and PT YM all displayed excellent reliability (ICC $\geq$ 0.94), whilst PT elongation displayed good reliability (ICC = 0.86). No systematic differences were present for any variable.

Table 4-1 The reliability of using B-mode ultrasound to measure VL properties at rest and the reliability of the optimal angle of force production derived from maximal isometric voluntary contraction in males and females (n = 36).

	Visit 1	Visit 2	Visit 3	*P*	TE (95% CI)	CV (95% CI)	ICC
VL CSA (cm^2)	29.0 ± 6.4	29.2 ± 6.0	29.1 ± 6.1	0.529	0.5 (0.5 – 0.7)	2.3 (1.9 – 2.8)	0.99
VL FL (mm)	60.7 ± 9.8	61.2 ± 9.6	60.3 ± 9.6	0.034*	1.6 (1.4 – 2.0)	2.6 (2.2 – 3.2)	0.97
VL PA (deg)	19.7 ± 3.2	19.9 ± 3.1	19.6 ± 3.2	0.042*	0.5 (0.4 – 0.6)	2.5 (2.1 – 3.1)	0.98
VL Thickness (mm)	20.0 ± 3.5	20.1 ± 3.4	19.9 ± 3.4	0.056	0.5 (0.4 – 0.6)	2.3 (1.9 – 2.9)	0.98
Optimal Angle (deg)	74.9 ± 9.7	74.6 ± 7.3	75.6 ± 7.3	0.749	5.4 (4.6 - 6.7)	7.7 (6.5 - 9.7)	0.57

Data are presented as mean ± SD. VL = *vastus lateralis*; CSA = cross-sectional area; FL = fascicle length; PA = pennation angle; CI = confidence interval; *P* = repeated measures ANOVA; TE = typical error; CV = coefficient of variation; ICC = intraclass correlation coefficient; * $P < 0.05$ from ANOVA.

Table 4-2 The reliability of using B-mode ultrasound to measure patella tendon properties at rest and during maximal isometric voluntary contraction in males and females (n = 36).

	Visit 1	Visit 2	Visit 3	*P*	TE (95% CI)	CV (95% CI)	ICC
PT CSA (mm^2)	85.0 ± 12.3	85.2 ± 12.2	84.9 ± 12.4	0.267	0.8 (0.7 – 1.0)	1.0 (0.9 – 1.2)	1.00
PT Length (mm)	65.5 ± 7.1	65.6 ± 6.9	65.2 ± 6.9	0.397	1.3 (1.1 – 1.6)	2.0 (1.7 – 2.5)	0.97
Knee Extension MVC (N·m)	167.9 ± 79.0	174.7 ± 83.4	176.5 ± 80.4	0.068	16.2 (13.7 – 20.1)	9.1 (7.7 – 11.4)	0.96
Antagonist Co-activation (N·m)	9.4 ± 6.1	9.3 ± 5.9	9.2 ± 5.7	0.526	0.7 (0.6 – 0.9)	11.5 (9.7 – 14.5)	0.99
PT Elongation (mm)	7.4 ± 1.0	7.6 ± 1.2	7.6 ± 1.3	0.164	0.4 (0.4 – 0.6)	6.0 (5.0 – 7.4)	0.86
PT Force (N)	4436 ± 1602	4618 ± 1757	4664 ± 1768	0.087	432 (365 – 535)	9.8 (8.2 – 12.2)	0.94
PT Stiffness ($N \cdot mm^{-1}$)	1134.1 ± 410.5	1139.4 ± 413.8	1140.9 ± 433.8	0.830	46.5 (39.3 – 57.6)	4.2 (3.5 – 5.2)	0.99
PT YM (MPa)	786.0 ± 248.6	789.7 ± 264.8	784.3 ± 258.0	0.204	38.2 (32.3 – 47.3)	4.4 (3.7 – 5.5)	0.98

Data are presented as mean ± SD. PT = patella tendon; CSA = cross-sectional area; YM = Young's modulus; CI = confidence interval; *P* = repeated measures ANOVA; TE = typical error; CV = coefficient of variation; ICC = intraclass correlation coefficient; KE = Knee Extension; CoA = Co-activation

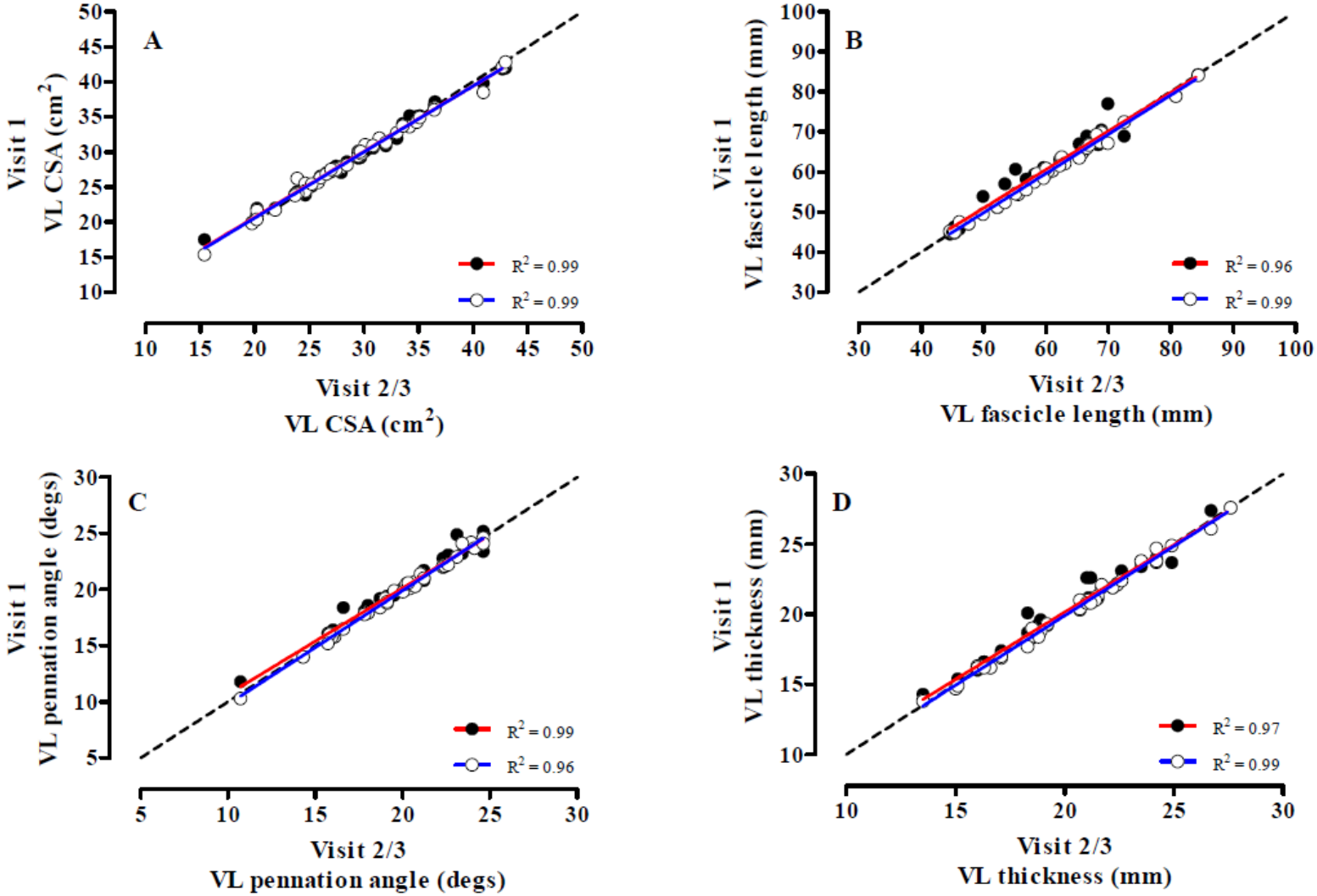

Figure 4-4 Reliability of using US to estimate resting measures of VL CSA (A), VL fascicle length (B), pennation angle (C) and VL thickness (D). The dashed line represents the line of equality. The solid red line denotes the regression line for visit 1 vs visit 2 and the solid blue line denotes the regression line for visit 1 vs visit 3. The black dots represent individual data points for visit 1 vs visit 2 and the white dots represent individual data points for visit 1 vs visit 3.VL = *vastus lateralis*, CSA = cross sectional area, US = ultrasound imaging. n = 36.

Vastus lateralis behaviour during eccentric exercise by repetition

Table 4-3 presents mean ± SD for each individual ECC repetition analysed. Individual data for absolute VL fascicle lengthening (FL at 110° knee angle – FL at 30° knee angle) are displayed in Figure 4-6, and relative VL fascicle lengthening (% increase from 30° (0%) to 110° (100%) knee angle) are displayed in Figure 4-7. CVs ranged from 2.7% to 3.5% and 2.9% to 3.7% for absolute and relative VL fascicle lengthening, respectively. Reliability for the three repetitions analysed were considered excellent (ICC ≥ 0.97) for both absolute and relative fascicle lengthening. Absolute fascicle lengthening for set 1 rep 5 was greater in visit 3 compared to visit 1 (0.9 ± 1.4 mm) and visit 2 (0.9 ± 1.7 mm). No other systematic differences were present for set 2 repetitions. CVs for ECC torque for each repetition ranged from 10.1% to 11.9%. Reliability was considered good (ICC ≥ 0.86) for set 2 reps 2 and 5, and excellent (ICC = 0.90) for set 1 rep 5.

Within- and between-set vastus lateralis behaviour

Table 4-4 presents mean ± SD for within and between set repetitions. Individual data points for within and between set fascicle lengthening values are presented in Figure 4-8. CVs for absolute fascicle lengthening were 1.2% and 1.1% for within and between set, respectively. CVs for relative fascicle lengthening were 1.2% for both within and between set. Reliability for both absolute and relative fascicle lengthening was considered excellent for both within and between set (ICC ≥ 0.99). No systematic differences were present for any within or between set measure. CVs for ECC torque were 8.3% and 9.0% for within and between set, respectively. Reliability for ECC torque was considered excellent (ICC ≥ 0.90) for within and between set.

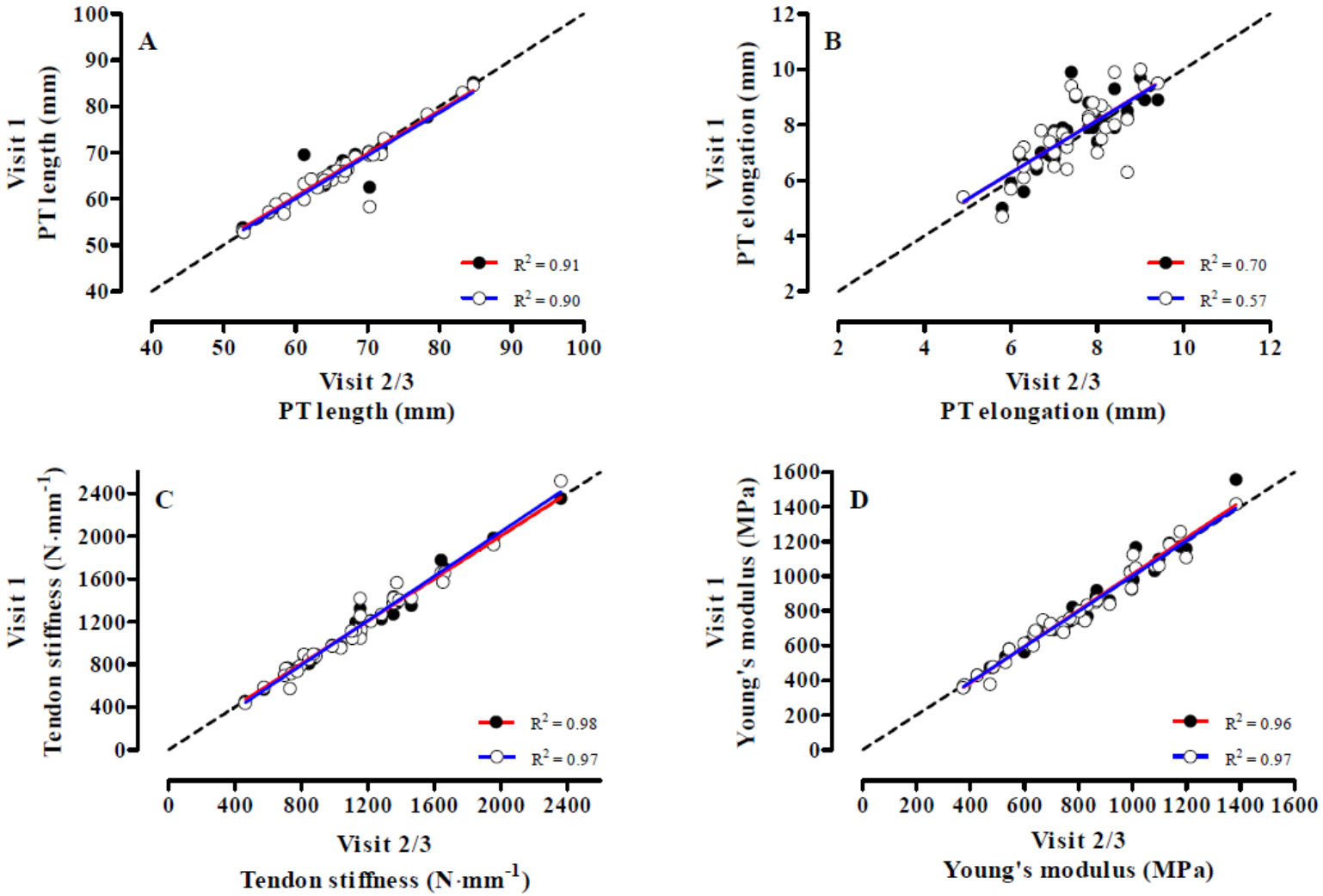

Figure 4-5 Reliability of estimating PT length at rest using US (A). Reliability of estimating PT elongation during a ramped iMVC using US (B). Reliability of estimating PT stiffness (C) and Young's modulus (D) during a ramped iMVC. The dashed line represents the line of equality. The solid red line denotes the regression line for visit 1 vs visit 2 and the solid blue line denotes the regression line for visit 1 vs visit 3. The black dots represent individual data points for visit 1 vs visit 2 and the white dots represent individual data points for visit 1 vs visit 3. PT = patella tendon, US = ultrasound imaging. n = 36.

Table 4-3 The reliability of using B-mode ultrasound to measure VL fascicle lengthening during maximal ECC exercise in males and females (n = 36).

	Visit 1	Visit 2	Visit 3	*P*	TE (95% CI)	CV (95% CI)	ICC
Set 1 Rep 5							
Absolute FL (mm)	44.2 ± 7.7	44.2 ± 7.8	45.1 ± 7.3	0.002*	1.2 (1.0 - 1.5)	3.1 (2.6 - 3.8)	0.98
Relative FL (%)	59.9 ± 14.5	59.5 ± 14.9	60.5 ± 14.4	0.059	1.9 (1.6 - 2.3)	3.7 (3.1 - 4.6)	0.97
ECC Torque (N·m)	1481.3 ± 456.8	1466.8 ± 437.6	1487.5 ± 475.5	0.838	149.8 (126.8 - 185.5)	10.1 (8.4 - 12.6)	0.90
Set 2 Rep 2							
Absolute FL (mm)	44.3 ± 7.5	44.5 ± 7.8	44.8 ± 7.2	0.360	1.4 (1.2 - 1.8)	3.5 (2.9 - 4.3)	0.97
Relative FL (%)	59.9 ± 14.7	59.9 ± 14.7	60.4 ± 14.4	0.448	1.9 (1.6 - 2.4)	3.6 (3.0 - 4.5)	0.98
ECC Torque (N·m)	1474.8 ± 450.7	1455.3 ± 446.4	1424.6 ± 446.6	0.405	156.7 (132.6 - 194.1)	11.3 (9.5 - 14.2)	0.88
Set 2 Rep 5							
Absolute FL (mm)	44.5 ± 7.7	44.6 ± 7.6	45.0 ± 7.1	0.198	1.1 (1.0 - 1.4)	2.7 (2.3 - 3.4)	0.98
Relative FL (%)	59.9 ± 14.8	60.0 ± 14.6	60.7 ± 14.4	0.147	1.5 (1.3 - 1.9)	2.9 (2.4 - 3.6)	0.99
ECC Torque (N·m)	1480.1 ± 473.1	1475.0 ± 463.8	1394.9 ± 401.0	0.143	172.9 (146.3 - 214.1)	11.9 (10.0 - 15.0)	0.86

Data are presented as mean ± SD. FL = fascicle lengthening; ECC = eccentric; YM = Young's modulus; CI = confidence interval; *P* = repeated measures ANOVA; TE = typical error; CV = coefficient of variation; ICC = intraclass correlation coefficient; * $P < 0.05$ from ANOVA

Table 4-4 The reliability of using B-mode ultrasound to measure within- and between-set VL fascicle lengthening during maximal ECC exercise in males and females (n = 36).

			P	TE (95% CI)	CV (95% CI)	ICC
Within Set	Set 2 Rep 2	Set 2 Rep 5				
Absolute FL (mm)	44.6 ± 7.4	44.7 ± 7.4	0.311	0.5 (0.4 - 0.6)	1.2 (1.0 - 1.5)	0.99
Relative FL (%)	60.1 ± 14.5	60.2 ± 14.5	0.652	0.7 (0.5 - 0.8)	1.2 (0.9 - 1.5)	1.00
ECC Torque (N·m)	1465.7 ± 439.8	1435.7 ± 419.0	0.330	128.9 (104.6 - 168.2)	8.3 (6.7 - 10.9)	0.91
Between Set	Set 1 Rep 5	Set 2 Rep 5				
Absolute FL (mm)	44.5 ± 7.5	44.7 ± 7.4	0.058	0.4 (0.4 - 0.6)	1.1 (0.9 - 1.4)	1.00
Relative FL (%)	60.0 ± 14.5	60.2 ± 14.5	0.175	0.6 (0.5 - 0.8)	1.2 (1.0 - 1.6)	1.00
ECC Torque (N·m)	1478.7 ± 450.4	1435.7 ± 419.0	0.206	141.6 (114.9 - 184.8)	9.0 (7.2 - 11.9)	0.90

Data are presented as mean ± SD. FL = fascicle lengthening; ECC = eccentric; CI = confidence interval; *P* = paired sample t-test; TE = typical error; CV = coefficient of variation; ICC = intraclass correlation coefficient.

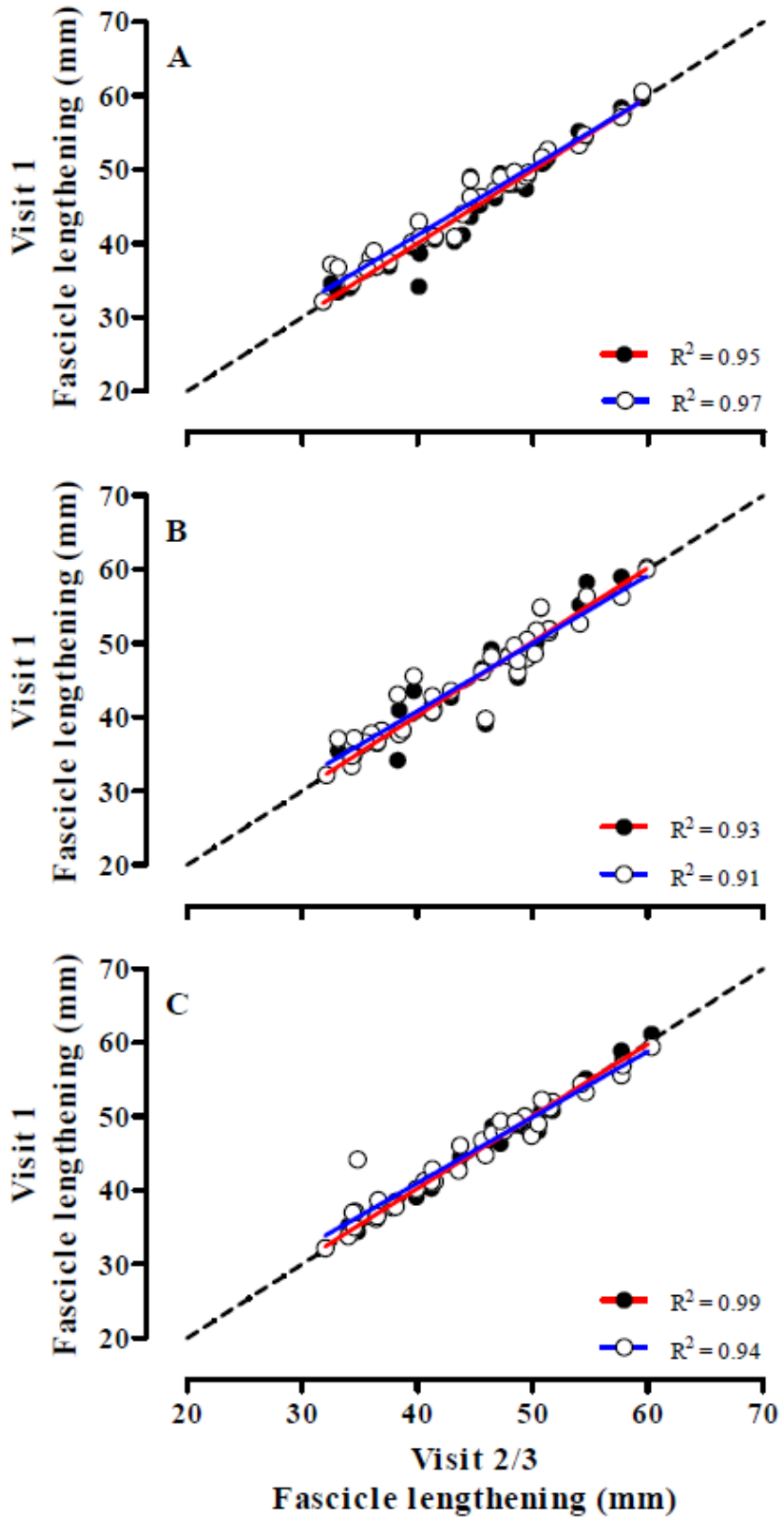

Figure 4-6 Reliability of measuring absolute fascicle lengthening during ECC exercise using US in set 1 rep 5 (A) Set 2 rep 2 (B) and set 2 rep 5 (C). The dashed line represents the line of equality. The solid red line denotes the regression line for visit 1 vs visit 2 and the solid blue line denotes the regression line for visit 1 vs visit 3. The black dots represent individual data points for visit 1 vs visit 2 and the white dots represent individual data points for visit 1 vs visit 3. VL = *vastus lateralis*, US = ultrasound imaging. n = 36.

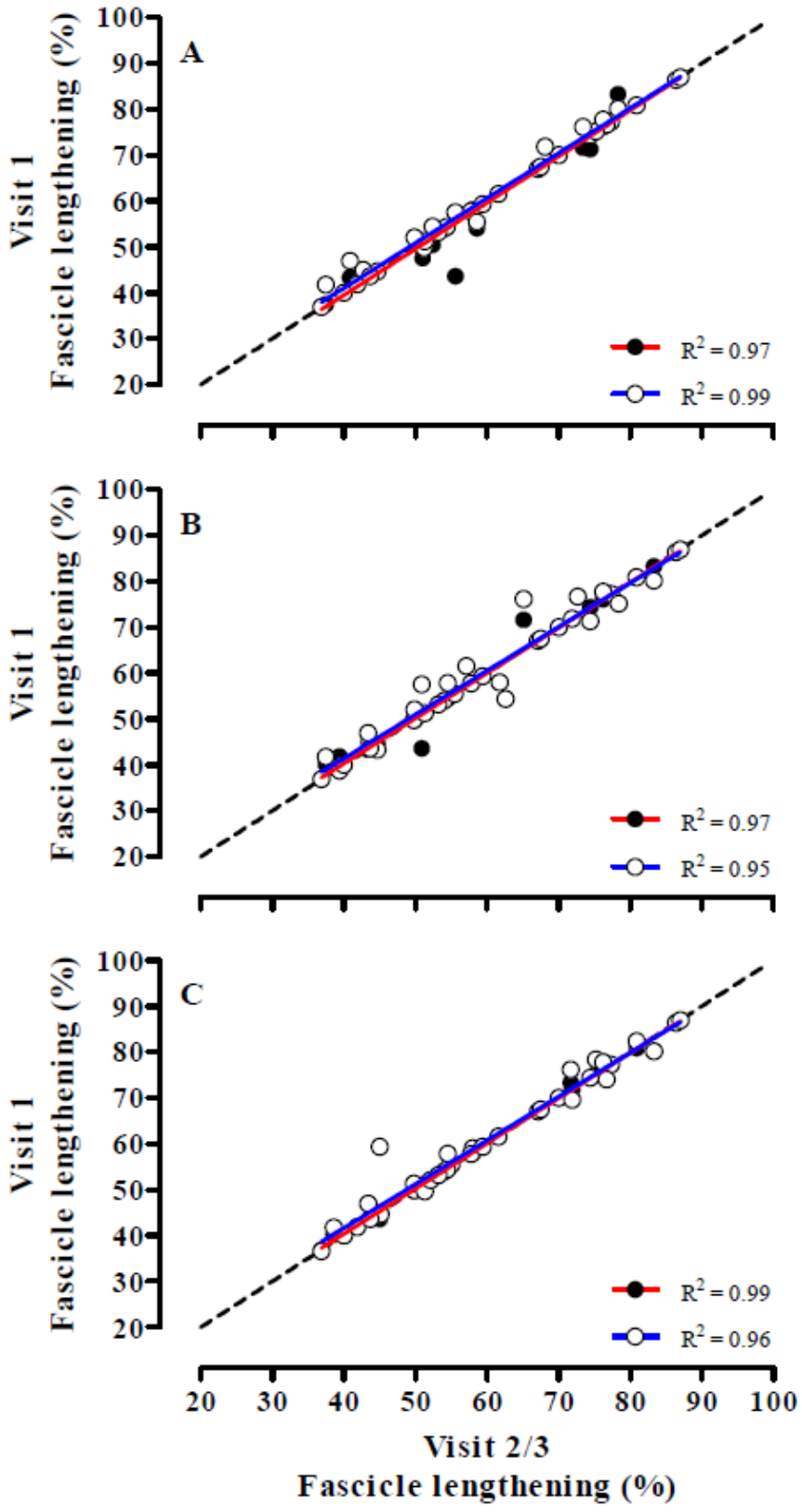

Figure 4-7. Reliability of measuring relative fascicle lengthening during ECC exercise using US in set 1 rep 5 (A) Set 2 rep 2 (B) and set 2 rep 5 (C). The dashed line represents the line of equality. The solid red line denotes the regression line for visit 1 vs visit 2 and the solid blue line denotes the regression line for visit 1 vs visit 3. The black dots represent individual data points for visit 1 vs visit 2 and the white dots represent individual data points for visit 1 vs visit 3. VL = *vastus lateralis*, US = ultrasound imaging. n = 36.

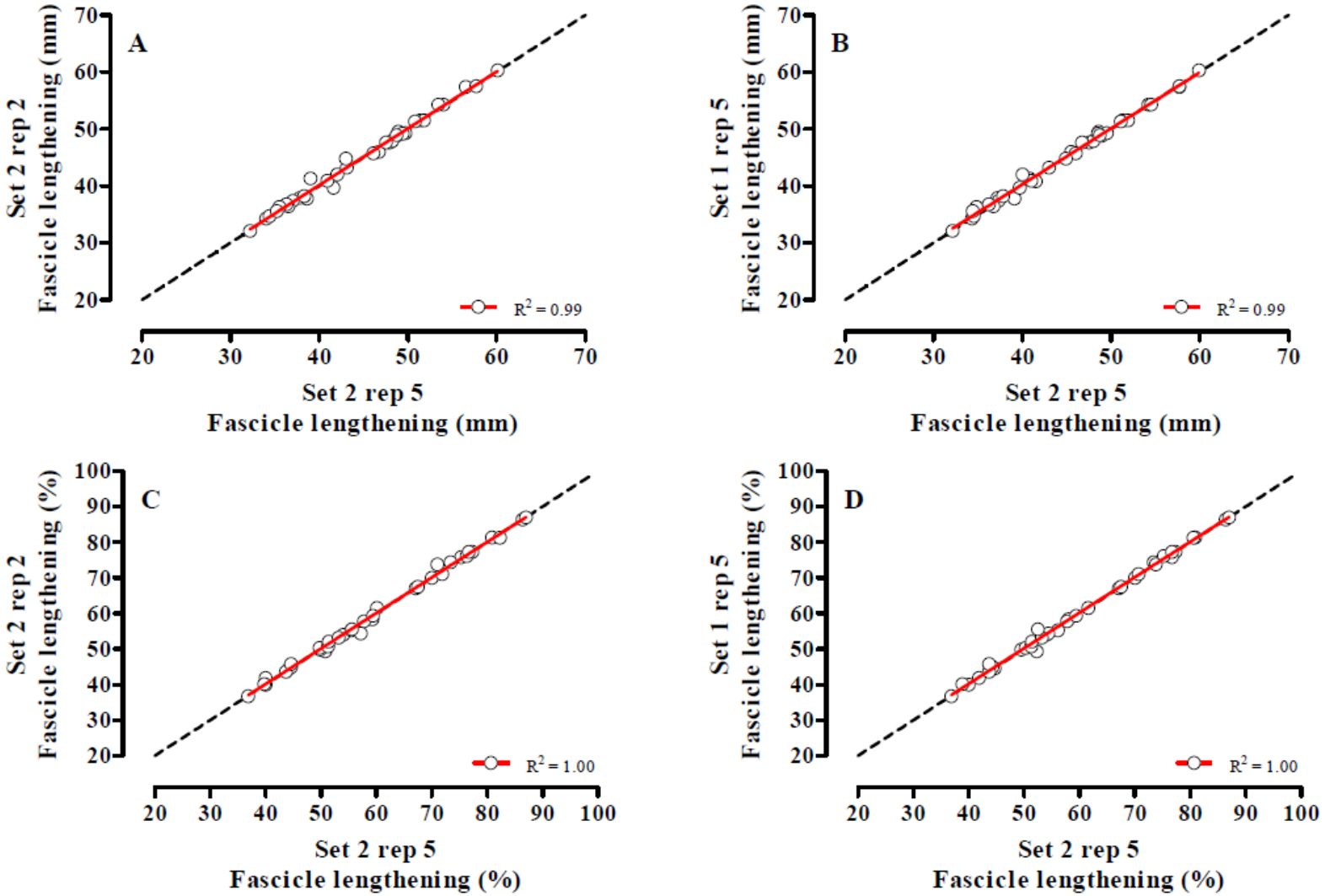

Figure 4-8 Reliability of estimating absolute VL fascicle lengthening during ECC exercise using US within- (A) and between-set (B). Reliability of estimating relative VL fascicle lengthening during ECC exercise using US within- (C) and between-set (D). The dashed line represents the line of equality. The solid red line denotes the regression line. VL = *vastus lateralis*, US = ultrasound imaging. n = 36.

Patella tendon property reliability by group

Table 4-5 presents the mean ± SD for patella properties by group. Figure 4-9 displays the individual data points for PT stiffness and Young's modulus by group. For all PT property measures, CVs ranged from 0.9% to 15.3% in the male group, 0.7% to 12.4% in the OCP group and 0.9% to 8.3% in the eumenorrheic group. For the male group, PT CSA, PT length, knee extension MVC, knee extension antagonist co-activation, PT stiffness and PT YM all showed excellent reliability (ICC ≥ 0.94) whilst PT elongation showed good reliability (ICC = 0.86). For the OCP group, PT CSA, knee extension MVC, knee extension antagonist co-activation, PT stiffness and PT YM showed excellent reliability (ICC ≥ 0.94) whilst PT length and PT elongation showed good reliability (ICC ≥ 0.84). For the eumenorrheic group, all measures showed excellent reliability (ICC ≥ 0.91). No systematic differences were present.

Vastus lateralis behaviour during eccentric exercise by repetition, by group

Table 4-6 presents mean ± SD for VL behaviour lengthening during ECC exercise. All measures, for all groups, showed excellent reliability (ICC ≥ 0.95). For the male group, CVs ranged from 4.2% to 4.7% for absolute fascicle lengthening and 4.6% to 5.6% for relative fascicle lengthening. For the OCP group, CVs ranged from 1.3% to 2.8% for absolute fascicle lengthening and 0.7% to 2.3% for relative fascicle lengthening. For the eumenorrheic group, CVs ranged from 2.0% to 2.6% for absolute fascicle lengthening and 2.0% to 2.7% for relative fascicle lengthening. No systematic differences were present for the male and OCP groups. In the eumenorrheic group, results from the ANOVA revealed a difference between visits for set one repetition five. Post *hoc* analysis showed that fascicle lengthening for set one repetition five was 1.5 ± 1.6 mm (absolute, $P = 0.007$) and 1.9 ± 2.0% (relative, $P = 0.009$) greater in visit three in comparison to visit one, and 1.3 ± 1.2 mm (absolute, $P = 0.003$) greater in visit

three in comparison to visit two, with no difference in relative fascicle lengthening between visit three and visit two.

Within- and between-set vastus lateralis behaviour by group

Figure 4-10 displays the individual data points for within- and between-day, absolute and relative fascicle lengthening by group. For within- and between-set fascicle lengthening, all measures for all groups showed excellent reliability (ICC $\geq$ 0.98) and CVs remained $\leq$ 1.6%. For the eumenorrheic group, between set fascicle lengthening was greater in set 2 than set 1 for both absolute (0.5 ± 0.7 mm, P = 0.024) and relative (0.8 ± 1.0%, P = 0.021) measures. No other systematic differences between measures were present.

Serum levels of estradiol and progesterone

There were no differences in estradiol levels in eumenorrheic females between visit 1 and visit 3 (124.9 ± 42.7 vs 118.0 ± 35.4 pg·ml^{-1}, respectively, P = 0.197). There were no differences in progesterone levels in eumenorrheic females between visit 1 and visit 3 (57.3 ± 20.9 vs 55.0 ± 18.6 ng·ml^{-1}, respectively, P = 0.197). All eumenorrheic females reported a positive ovulation result.

Table 4-5 The reliability of using B-mode ultrasound to measure patella tendon properties at rest and during maximal isometric voluntary contraction by group (males, n = 12; OCP females n = 12, eumenorrheic females, n = 12).

		Visit 1	Visit 2	Visit 3	*P*	TE (95% CI)	CV (95% CI)	ICC
PT CSA (mm^2)	Males	97.1 ± 11.9	97.2 ± 11.7	97.1 ± 11.8	0.718	0.9 (0.7 - 1.4)	0.9 (0.7 - 1.4)	1.00
	OCP	79.5 ± 8.5	79.6 ± 8.6	79.3 ± 8.8	0.645	0.7 (0.5 - 1.0)	0.9 (0.7 - 1.4)	1.00
	Eumenorrheic	78.4 ± 5.1	78.8 ± 5.1	78.2 ± 5.3	0.287	0.9 (0.7 - 1.4)	1.2 (0.9 - 0.8)	0.98
PT Length (mm)	Males	68.6 ± 8.5	68.7 ± 8.5	68.1 ± 8.5	0.082	0.7 (0.5 - 1.0)	1.0 (0.8 - 1.6)	0.99
	OCP	63.8 ± 5.0	64.0 ± 4.5	63.4 ± 4.6	0.778	2.1 (1.6 - 3.1)	3.2 (2.4 - 4.9)	0.84
	Eumenorrheic	64.1 ± 6.7	64.0 ± 6.5	64.1 ± 6.7	0.998	0.6 (0.4 - 0.9)	0.9 (0.7 - 1.4)	0.99
KE MVC (N·m)	Males	250.0 ± 77.2	260.5 ± 78.8	251.1 ± 74.4	0.382	21.6 (16.3 – 32.5)	8.2 (6.1 – 12.6)	0.94
	OCP	138.8 ± 41.9	141.4 ± 47.3	151.8 ± 62.3	0.138	13.7 (10.3 – 20.1)	11.0 (8.2 – 17.1)	0.94
	Eumenorrheic	114.8 ± 28.2	122.3 ± 38.2	126.6 ± 41.3	0.065	10.8 (8.2 – 16.3)	8.0 (6.0 - 12.4)	0.93
KE Antagonist CoA (N·m)	Males	10.6 ± 7.6	10.2 ± 7.4	10.4 ± 7.3	0.405	0.7 (0.6 – 1.1)	15.3 (11.4 – 24.0)	0.99
	OCP	8.9 ± 5.5	8.9 ± 5.2	8.9 ± 5.0	0.943	0.8 (0.6 – 1.2)	11.2 (8.3 – 17.4)	0.98
	Eumenorrheic	8.7 ± 5.1	8.7 ± 5.2	8.4 ± 4.8	0.289	0.6 (0.4 – 0.9)	7.1 (5.3 – 10.8)	0.99
PT Elongation (mm)	Males	7.3 ± 1.1	7.6 ± 1.3	7.5 ± 1.3	0.337	0.5 (0.4 - 0.8)	6.8 (5.1 - 10.6)	0.86
	OCP	7.6 ± 1.2	7.8 ± 1.4	7.8 ± 1.6	0.414	0.5 (0.4 - 0.8)	7.2 (5.4 - 11.0)	0.89
	Eumenorrheic	7.4 ± 0.8	7.5 ± 0.8	7.6 ± 0.9	0.301	0.3 (0.2 - 0.4)	3.5 (2.7 - 5.4)	0.91
PT Force (N)	Males	5915 ± 1499	6198 ± 1498	5994 ± 1558	0.374	491 (370 – 740)	8.3 (6.2 – 12.8)	0.92
	OCP	4161 ± 1164	4304 ± 1417	4527 ± 1742	0.255	476 (359 – 716)	12.4 (9.2 – 19.3)	0.91
	Eumenorrheic	3232 ± 713	3353 ± 1000	3471 ± 990	0.209	307 (232 – 463)	8.3 (6.2 – 12.7)	0.91
PT Stiffness ($N \cdot mm^{-1}$)	Males	1575.3 ± 330.3	1574.0 ± 345.9	1605.8 ± 343.4	0.343	59.0(44.5 - 88.8)	3.8 (2.9 - 5.8)	0.98
	OCP	1005.9 ± 215.3	1021.6 ± 225.0	1011.4 ± 236.1	0.651	40.1 (30.6 - 61.2)	3.8 (2.8 - 5.7)	0.97
	Eumenorrheic	821.0 ± 208.8	822.5 ± 209.4	805.6 ± 219.7	0.487	36.3 (27.4 – 54.7)	4.8 (3.6 – 7.4)	0.98
PT YM (MPa)	Males	1014.8 ± 207.5	1014.1 ± 246.2	1026.3 ± 210.6	0.242	50.2 (37.8 - 75.7)	4.5 (3.4 - 6.9)	0.96
	OCP	732.9 ± 159.8	747.1 ± 188.2	728.6 ± 164.6	0.368	35.3 (26.7 - 53.3)	4.0 (3.0 - 6.1)	0.97
	Eumenorrheic	610.3 ± 184.9	607.9 ± 184.9	597.9 ± 187.5	0.501	26.0 (19.6 – 39.1)	4.6 (3.5 – 7.0)	0.98

Data are presented as mean ± SD. PT = patella tendon; CSA = cross-sectional area; YM = Young's modulus; OCP = oral contraceptive pill; CI = confidence interval; *P* = repeated measures ANOVA; TE = typical error; CV = coefficient of variation; ICC = intraclass correlation coefficient; KE = Knee Extension; CoA = Co-activation

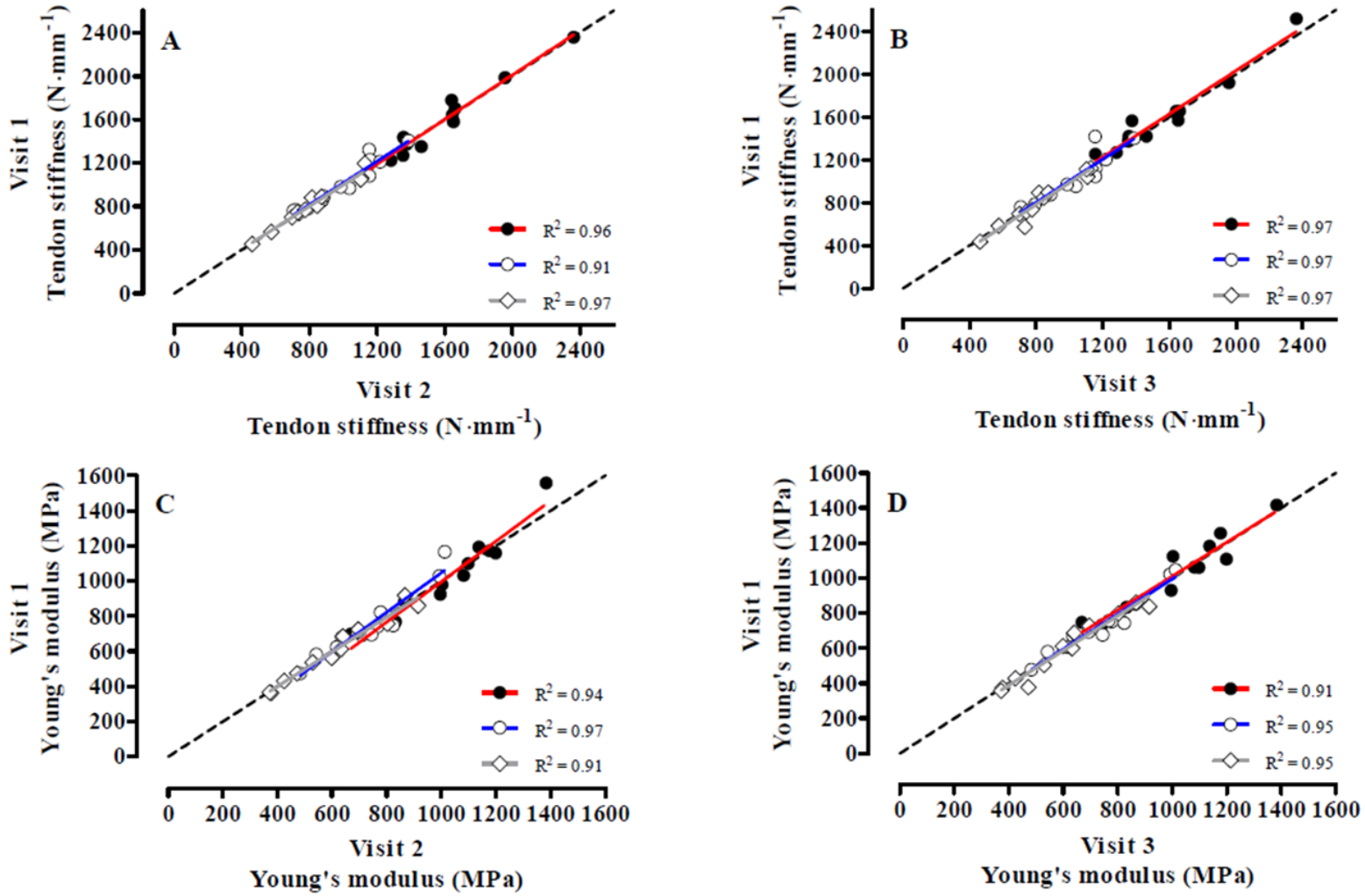

Figure 4-9 Reliability of estimating PT stiffness during a ramped iMVC by group (males, n = 12; OCP females n = 12, eumenorrheic females, n = 12) for visit 1 vs visit 2 (A) and visit 1 vs visit 3 (B). Reliability of estimating Young's modulus during a ramped iMVC for visit 1 vs visit 2 (C) and visit 1 vs visit 3 (D). The dashed line represents the line of equality. The solid red line denotes the regression line for males, the solid blue line denotes the regression line for OCP users, and the solid grey line denotes the regression line for eumenorrheic females. The black dots represent individual data points for males, the white dots represent individual data points for OCP users, and the white diamonds represent the individual data points for eumenorrheic females. PT = patella tendon, US = ultrasound imaging, OCP = oral contraceptive pill.

Table 4-6 The reliability of using B-mode ultrasound to measure VL fascicle lengthening during maximal ECC exercise by group (males, n = 12; OCP females n = 12, eumenorrheic females, n = 12).

		Visit 1	Visit 2	Visit 3	*P*	TE (95% CI)	CV (95% CI)	ICC
Set 1 Rep 5								
Absolute FL (mm)	Males	45.6 ± 7.3	45.6 ± 7.9	46.6 ± 6.8	0.252	1.8 (1.4 - 2.8)	4.7 (3.5 - 7.2)	0.95
	OCP	42.5 ± 7.9	42.3 ± 7.8	42.6 ± 7.7	0.477	0.7 (0.5 - 1.0)	1.5 (1.1 - 2.3)	0.99
	Eumenorrheic	44.5 ± 8.3	44.7 ± 8.0	46.0 ± 7.3	0.002*	0.9 (0.7 - 1.3)	2.3 (1.7 - 3.5)	0.99
Relative FL (%)	Males	59.2 ± 13.9	58.1 ± 14.5	59.5 ± 13.6	0.296	2.7 (2.0 - 4.1)	5.6 (4.2 - 8.5)	0.97
	OCP	56.8 ± 14.7	56.2 ± 14.4	56.6 ± 14.7	0.325	1.0 (0.7 - 1.5)	1.6 (1.2 - 2.5)	1.00
	Eumenorrheic	63.6 ± 15.4	64.2 ± 15.8	65.5 ± 14.5	0.020*	1.6 (1.2 - 2.4)	2.8 (2.1 - 4.2)	0.99
Set 2 Rep 2								
Absolute FL (mm)	Males	45.6 ± 7.2	45.9 ± 7.9	45.9 ± 6.9	0.891	1.9 (1.4 - 2.9)	4.8 (3.6 - 7.3)	0.95
	OCP	42.7 ± 7.9	42.6 ± 8.1	42.5 ± 7.5	0.897	1.2 (0.9 - 1.8)	2.8 (2.1 - 4.3)	0.98
	Eumenorrheic	44.7 ± 7.8	45.2 ± 7.7	46.1 ± 7.2	0.069	1.1 (0.8 - 1.7)	2.6 (1.9 - 3.9)	0.98
Relative FL (%)	Males	58.6 ± 13.9	58.3 ± 14.4	59.1 ± 14.2	0.357	2.6 (1.9 - 3.8)	5.2 (3.9 - 8.0)	0.97
	OCP	57.1 ± 14.8	56.6 ± 14.7	56.3 ± 14.3	0.416	1.3 (1.0 - 2.0)	2.3 (1.7 - 3.4)	0.99
	Eumenorrheic	63.9 ± 15.7	64.8 ± 15.1	65.8 ± 14.3	0.142	1.7 (1.2 - 2.5)	2.7 (2.0 - 4.1)	0.99
Set 2 Rep 5								
Absolute FL (mm)	Males	45.8 ± 7.7	46.0 ± 7.8	46.1 ± 6.5	0.771	1.7 (1.3 - 2.6)	4.2 (3.2 - 6.5)	0.96
	OCP	42.3 ± 7.9	42.4 ± 7.8	42.7 ± 7.7	0.298	0.5 (0.4 - 0.8)	1.3 (1.0 - 1.9)	1.00
	Eumenorrheic	45.3 ± 7.9	45.4 ± 7.5	46.1 ± 7.1	0.077	0.9 (0.7 - 1.4)	2.0 (1.5 - 3.1)	0.99
Relative FL (%)	Males	58.4 ± 14.3	58.3 ± 14.4	59.6 ± 13.6	0.377	2.3 (1.8 - 3.5)	4.6 (3.5 - 7.0)	0.98
	OCP	56.6 ± 14.7	56.6 ± 14.7	56.6 ± 14.7	0.866	0.4 (0.3 - 0.5)	0.7 (0.5 - 1.1)	1.00
	Eumenorrheic	64.7 ± 15.4	65.1 ± 14.6	65.8 ± 14.4	0.129	1.2 (0.9 - 1.8)	2.0 (1.5 - 3.0)	1.00

Data are presented as mean ± SD. FL = fascicle lengthening; OCP = oral contraceptive pill; CI = confidence interval; *P* = repeated measures ANOVA; TE = typical error; CV = coefficient of variation; ICC = intraclass correlation coefficient; * $P < 0.05$ from ANOVA

Table 4-7 The reliability of using B-mode ultrasound to measure within- and between-set VL fascicle lengthening during maximal ECC exercise by group (males, n = 12; OCP females n = 12, eumenorrheic females, n = 12).

		Set 2 Rep 2	Set 2 Rep 5	*P*	TE (95% CI)	CV (95% CI)	ICC
Within Set							
Absolute FL (mm)	Males	45.8 ± 7.2	46.0 ± 7.2	0.471	0.6 (0.4 - 1.0)	1.4 (1.0 - 2.3)	0.98
	OCP	42.6 ± 7.8	42.5 ± 7.8	0.830	0.5 (0.3 - 0.8)	1.2 (0.8 - 2.0)	1.00
	Eumenorrheic	45.3 ± 7.5	45.6 ± 7.5	0.130	0.4 (0.3 - 0.7)	1.0 (0.7 - 1.7)	1.00
Relative FL (%)	Males	57.8 ± 14.0	57.8 ± 13.9	0.374	0.6 (0.4 - 0.9)	1.2 (0.9 - 2.1)	1.00
	OCP	56.7 ± 14.6	56.6 ± 14.7	0.822	0.7 (0.5 - 1.1)	1.2 (0.8 - 2.0)	1.00
	Eumenorrheic	64.8 ± 15.0	65.2 ± 14.7	0.225	0.7 (0.5 - 1.2)	1.1 (0.8 - 1.9)	1.00
Between Set		Set 1 Rep 5	Set 2 Rep 5				
Absolute FL (mm)	Males	46.0 ± 7.2	46.0 ± 7.2	0.919	0.4 (0.3 - 0.6)	0.9 (0.6 - 1.5)	0.99
	OCP	42.5 ± 7.8	42.5 ± 7.8	0.273	0.3(0.2 - 0.5)	0.7 (0.5 - 1.2)	1.00
	Eumenorrheic	45.1 ± 7.8	45.6 ± 7.5	0.024*	0.5 (0.4 - 0.9)	1.3 (0.9 - 2.3)	1.00
Relative FL (%)	Males	57.8 ± 13.9	58.8 ± 13.9	0.192	0.7 (0.5 - 1.1)	1.3 (0.9 - 2.2)	1.00
	OCP	56.5 ± 14.5	56.6 ± 14.7	0.686	0.3 (0.2 - 0.6)	0.6 (0.4 - 1.0)	1.00
	Eumenorrheic	64.4 ± 15.2	65.2 ± 14.7	0.021*	0.7 (0.5 - 1.2)	1.4 (1.0 - 2.4)	1.00

Data are presented as mean ± SD. FL = fascicle lengthening; OCP = oral contraceptive pill; CI = confidence interval; *P* = repeated measures ANOVA; TE = typical error; CV = coefficient of variation; ICC = intraclass correlation coefficient; * $P < 0.05$ from ANOVA

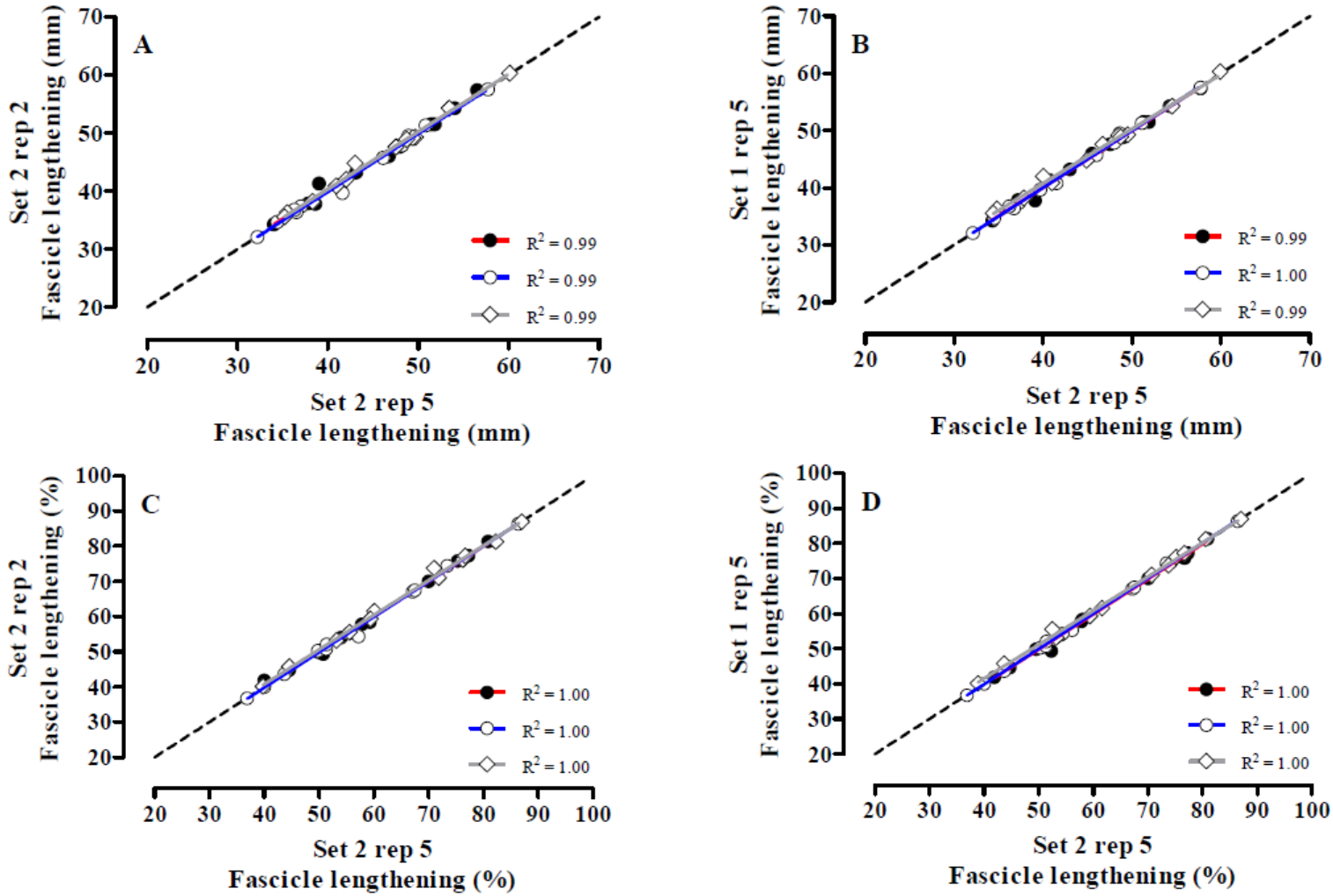

Figure 4-10 Reliability of estimating absolute VL fascicle lengthening during ECC exercise using US by group (males, n = 12; OCP females n = 12, eumenorrheic females, n = 12) within- (A) and between-set (B). Reliability of estimating relative VL fascicle lengthening during ECC exercise using US by group within- (C) and between-set (D). The dashed line represents the line of equality. The solid red line denotes the regression line for males, the solid blue line denotes the regression line for OCP users, and the solid grey line denotes the regression line for eumenorrheic females. The black dots represent individual data points for males, the white dots represent individual data points for OCP users, and the white diamonds represent the individual data points for eumenorrheic females. VL = *vastus lateralis*, US = ultrasound imaging, OCP = oral contraceptive pill.

4-4 DISCUSSION

The results from chapter three in this thesis showed that US is a valid and reliable tool for measuring PT CSA. Additionally, chapter three showed that the operator who was to perform all subsequent US analysis in the thesis could produce reliable estimates of PT CSA at rest. These findings from chapter three supported the use of US as a measurement tool in the current study. One of the main aims of this thesis was to determine the difference in patella tendon stiffness, VL muscle fascicle lengthening, and indirect markers of muscle damage over two bouts of maximal ECC exercise of the knee extensors, separated by four weeks. Therefore, the use of US needed to be expanded from measuring PT CSA at rest, to measures of the VL at rest and measures of the PT and VL during dynamic activity, across time. Therefore, the main aim of chapter 4 was to determine whether 2D B-mode ultrasound is a reliable imaging method for estimating PT properties both at rest and during maximal isometric exercise, and VL properties both at rest and during maximal ECC exercise, across a seven-day and four-week period. Additionally, another aim of the thesis was to compare the responses of males, OCP using females, and eumenorrheic females to maximal ECC knee extension exercise. By extension, a secondary aim of this study was to assess the reliability of US imaging as method for estimating PT and VL properties both at rest and during maximal exercise in males, OCP using females, and eumenorrheic females. One of the main findings from this study is that US is a reliable technique for measuring resting properties of the PT and VL at rest and during exercise with both one and four weeks between measures. The current study also showed that both PT and VL properties can be reliably measured using US for males, OCP users and eumenorrheic females.

Resting patella tendon properties

As concluded from chapter three in this thesis, and from previous research, US was found to be a valid and reliable measure of PT CSA (Stenroth et al., 2019, Gellhorn and Carlson, 2013). Additionally, chapter three showed that there was excellent inter-rater reliability in measuring PT CSA using US, highlighting the competence of the rater who subsequently performed the US analysis in the current study. One difference between the current study and the previous chapter was the length of time between measurements. In chapter 3, only a three-day period was observed between measures, ensuring minimal physiological variation between visits. In the present study, a one- and four-week time period was allowed between measures, to account for the fact that four weeks would be the test-re-test period between experimental visits in chapter five, and the follow-up measures will persist for one week following exercise. Despite a longer period between retest occasions the results of the present study, in comparison to chapter three, displayed slightly better absolute (TE 0.8 mm vs 1.4 mm) and relative (CV 1.0% vs 1.6%) reliability, with both studies displaying similar ICCs (1.00 vs 0.99). The high reliability of using US to estimate PT CSA in chapter three in relation to the previous studies (Stenroth et al., 2019, Ekizos et al., 2013) was discussed in detail in section 3-4. The slightly improved reliability in the present study could be due to the examiner performing the measurement and analysis becoming more experienced over time, which has been shown to increase reliability (Dudley-Javoroski et al., 2010), which could explain why the mean TE and CVs in the current study were lower than the 95% confidence intervals present for rater 1 in chapter three (TE 1.8 – 3.5 mm^2, CV 2.0 – 4.0%).

Patella tendon length and elongation during exercise

Patella tendon CSA typically consists of measuring the tendon at 25%, 50% and 75% of tendon length, with the mean of all three sites used to estimate whole tendon CSA (Hicks et al., 2013, Kongsgaard et al., 2007, Couppe et al., 2008, Couppe et al., 2016, Murtagh et al., 2018). It is therefore important that estimation of PT length is accurate so that these sites can be identified consistently. Gellhorn and Carlson (2013) reported an ICC of 0.96 for repeated measurements of PT length, which corroborated findings from the present study, which found an ICC of 0.97 for PT length, giving confidence in the correct identification of CSA measurement sites. In addition to resting measures, the correct quantification of tendon properties during exercise is required to properly estimate tendon stiffness and Young's Modulus, specifically, PT elongation. Onambele et al. (2007) reported excellent reliability (ICC = 0.91) for measuring PT elongation using US, which was slightly higher than the present study which displayed good reliability (ICC = 0.86). One reason why PT elongation might not be as reliable as the study by Onambele et al. (2007) is due to the variation in PT force during the ramped iMVC (CV 9.8%). As tendon elongation is dependent on the amount of force applied (Hansen et al., 2006), this variation in tendon force might have affected the amount of PT elongation experienced between each trial. Alternatively, there is a possibility that the ultrasound was slightly misaligned longitudinally during ramped iMVC, which has been shown to produce an overestimation of tendon elongation (Magnusson et al., 2001). However, as tendon elongation is a precursor to tendon stiffness and YM calculation (Onambele et al., 2007) as well as being sensitive to the amount of force applied, the reliability of this measure should not receive as much focus as the downstream calculations, which, in the present study, were deemed excellent, as discussed in the next section.

Patella tendon stiffness and Young's modulus

Despite the fluctuations in knee extension MVC, antagonist co-activation, PT force and elongation, reliability of PT stiffness and YM was excellent (ICC ≥ 0.98). The main reason for this is most likely that for each participant, each measure of stiffness and YM was made relative to the visit that produced the lowest force. Whilst no between participant comparisons were made during this study, this method of using force corresponding to the weakest contraction has been used in the literature to account for differences in force production between participants and allow comparisons to be made (Onambele et al., 2007, Hicks et al., 2013, Hicks et al. 2017). As PT stiffness and YM was standardised to the iMVC of the weakest contraction per participant, it is unsurprising that reliability was excellent, once PT elongations and force fluctuations had been eliminated. Chapter five will investigate measures of PT stiffness and YM prior to two bouts of maximum ECC exercise. The results of the current study show that these measures of tendon properties do not change following exposure to low volumes of ECC contractions. This provides confidence that any changes detected in PT stiffness and YM in chapter five are not due to normal biological variation or measurement error.

Resting measures of the vastus lateralis

Ultrasound measurements of VL dimensions (CSA and thickness) in the present study were shown to have excellent reliability (ICC ≥ 0.98) which corroborates findings from Reeves et al. (2004), who reported ICCs of 0.997 – 0.999 and TE ranging from 0.15 cm^2 to 0.40 cm^2, which equated to 1.0% and 3.3%, when presented as a percentage, similar to the present study (2.3%). This finding is of particular importance when conducting sex differences research on the muscle damage response to exercise. For example, CK has been normalised to VL CSA

when comparing the response of males and females to ECC exercise (Hicks et al., 2016), to account for large differences in muscle volume and force production between groups. Moreover, normalising ECC torque during exercise to VL CSA is also a method utilised in sex differences research (Morawetz et al., 2019). Therefore, the high reliability of measuring VL CSA shown in the current study allows these 'normalising' techniques to be employed with confidence in future studies.

Vastus lateralis muscle architecture at rest (FL, PA and thickness) in the current study showed excellent reliability between measures taken one and four weeks apart (ICCs, ≥ 0.97), which is similar to that reported by Alegre et al. (2006), who reported ICCs of 0.95, 0.96 and 0.96 for FL, PA, and thickness, respectively. Changes in resting muscle architecture are commonly used to measure training adaptations, such as increases in FL following 12 weeks of ECC based training (Baroni et al., 2013). It is therefore crucial that highly reliable imaging techniques are used to suitably quantify these architectural measures, avoiding inaccurate estimation of changes in these measures as a result of an exercise intervention (Klimstra et al., 2007). In the present study, both FL and PA were systematically different between visits 1 and 3, despite the small CVs (2.6% and 2.5%, respectively) and excellent reliability (ICC ≥ 0.97). Although the differences for FL and PA were small (0.4 ± 0.9 mm and 0.1 ± 0.3°, respectively), an explanation for this difference might be due to slight probe rotation in one or both visits, affecting the quantification of each measure (Klimstra et al., 2007, Kawakami et al., 1995). It is also possible that in the four-week time period between measures, some training-induced architectural differences might have occurred, as the participants were actively involved in training between visits. However, as FL was shorter on the four-week follow up, this is unlikely as resistance training tends to increase, not shorten FL (Baroni et al., 2013, Blazevich, 2006, Alegre et al., 2006). Nevertheless, the systematic differences between visits one and three were

small and although this will have increased TE (Hopkins, 2000), the systematic bias and CV were low. Overall, it can be concluded that US provides a highly reliable method of estimating VL properties at rest.

Optimal angle of force production

In the present study, the reliability of OA measurements was moderate (ICC = 0.57), with a TE of 5.4° and CV of 7.7%. Shifts in OA towards a longer muscle length are indicative of EIMD (Hicks et al., 2016, Chen et al., 2013), most commonly attributed to an addition of sarcomeres in series following a remodelling of the muscle from damage elicited by ECC exercise (Proske and Morgan, 2001). However, little data on the reliability of OA is present in the literature. Recently, Oranchuk et al. (2020) investigated OA in the knee extensors over three occasions and reported a CV of 8.0% and ICC of 0.58 between visits one and two and a CV of 7.3% and ICC of 0.64 between visits two and three, which is similar to the current study. Chen et al. (2013) reported a CV of 6.7% and an *r* value of 0.96 for the test-retest reliability of OA measures in the upper arm, although it was not stated how many participants or contractions this was derived from.

However, despite the differences in muscle architecture between the upper arm and VL, the similar CVs in the studies by Chen et al. (2013) and Oranchuk et al. (2020) to the present study indicates some consistency between the literature. It must be noted that both Chen et al. (2013) and Oranchuk et al. (2020) used concentric contractions to determine OA to the nearest degree, in contrast to repeated isometric contractions in the present study. As the current study used 5° intervals in a range of 60 - 90°, the precise OA could not be measured. No literature currently exists comparing isometric and concentric methods of obtaining OA, so it is difficult to say which is the superior method, but the similar reliability between isometric and concentric

measures of OA indicate that either method can be used. However, the ICC of 0.57 and the TE of 5.4° in the present study requires some caution when interpreting shifts in OA as a marker of EIMD, specifically when compared over multiple time-points.

Vastus lateralis fascicle lengthening during maximal ECC exercise

Although the present study is the first comprehensive study on the reliability of US derived VL fascicle lengthening during maximal isokinetic ECC exercise, similar reliability analyses were reported by Hicks et al. (2013) and Guilhem et al. (2011). These analyses were conducted on a sub-sample of five people, where ICCs of 0.99 and 0.82 were reported, respectively. It must be noted that the high reliability displayed by Hicks et al. (2013) might be due to the small sample size in comparison to the present study, which can affect estimates of error (Springate, 2011). Nevertheless, US derived VL fascicle lengthening reliability has twice been investigated by Penailillo et al. (2015, 2016), although these studies were conducted using a sub-maximal, ECC cycling protocol. Nonetheless, an ICC range of 0.97 to 0.98 (absolute fascicle lengthening) and 0.97 to 0.99 (relative fascicle lengthening) in the present study was similar to Penailillo et al. (2015), who reported an ICC of 0.99. Penailillo et al. (2016) reported a CV of 5.6% for fascicle lengthening, which was larger than the CV range of 2.7% to 3.5% reported in the present study. Both studies by Penailillo et al. (2015, 2016), the study by Hicks et al. (2013), and the present study used the linear extrapolation (Reeves and Narici, 2003) to partially estimate FL, which typically is associated with a 2-7% error (Finni et al., 2001, Finni et al., 2003). With this taken into consideration, it would appear that the greater proportion of the fascicle that needs to be estimated would lead to a greater chance for the error reported by Finni et al. (2003) to occur. Whilst the present study would be minimally affected by this estimation error, due to the range of motion being consistent between visits and therefore requiring similar levels of linear

extrapolation, FL comparisons between studies might be subject to this error, depending on the range of motion around the joint utilised in the procedure and the muscle architecture of the participants. For example, the high ICC reported by Hicks et al. (2013) in comparison to the present study and the studies by Penailillo et al. (2015, 2016), might be due to the estimations of FL at longer muscle lengths. Hicks et al. (2013) used a maximum knee angle of 90° in comparison to ~120° in the studies by Penailillo et al. (2015, 2016) and 110° in the present study, meaning that less of the fascicle length might have been estimated, therefore minimising the error associated with the linear continuation method (Finni et al., 2001, Finni et al., 2003). Although, the level of FL estimation is dependent on starting FL and the behaviour of the fascicle during exercise, which has been shown to differs between groups, specifically, males and females (Hicks et al., 2013, Hicks et al., 2016). However, due to different probe lengths (38 mm) used by Hicks et al. (2013) and the present study (50 mm), the increased range of motion might have been absorbed by the increased field of view, meaning a similar amount of FL was estimated for both studies, despite the differences in range of motion, leading to the similar reports of reliability.

An important feature of this study was to determine VL behaviour both within and between sets of ECC contractions. Typically, motor output is more variable during ECC contractions than CON contractions, as ECC contractions are likely more unaccustomed movements requiring different neural control (Christou and Carlton, 2002). Therefore, this study was designed so that participants could perform enough ECC contractions to be able to produce consistent force outputs and allow consistent measurement of fascicle behaviour, without performing too many ECC contractions that might cause excessive EIMD and alter MTU behaviour (Lau et al., 2015). Although CVs for ECC torque were 8.3% and 9.0% for within- and between-set, respectively, reliability was still considered excellent. This stable force output

during ECC exercise resulted in CVs ≤ 1.2% and ICCs ≥ 0.99, with no systematic bias present for both absolute and relative changes in FL, within- and between sets. This study therefore confirms that US is a reliable tool for estimating fascicle lengthening during maximal ECC exercise.

Measures of patella tendon and vastus lateralis properties for males, OCP using females and eumenorrheic females

Finally, the last aim of this study was to determine whether the reliability of US measures of PT and VL properties at rest and during maximal exercise were different between males, OCP users and eumenorrheic females, as these experimental groups will be investigated in chapter five. Reliability for PT CSA measures was excellent for all groups (ICC ≥ 0.98) with all CVs ≤ 1.2%. The main discrepancy discovered was the high CV (12.4%) presented in the OCP group for tendon elongation, although both males and eumenorrheic females displayed higher CVs (both 8.3%) than for other measures. However, ICCs ≥ 0.91 for all groups still indicate excellent reliability. Of particular importance is the similar reliability scores for relative PT stiffness and YM between groups. As the elastic properties of tendon can be a determinant of EIMD, acting as a mechanical buffer and reducing the length of muscle fascicle lengthening and subsequent EIMD magnitude (Hicks et al., 2017, Guilhem et al., 2016), and changes in tendon compliance might be a mechanism of the RBE (Hyldahl et al., 2017, Lau et al. 2015), the reliability of tendon properties in the absence of EIMD is important when implementing interventions that might alter these properties.

Reliability of fascicle lengthening was excellent for all groups (ICC ≥ 0.95) with CVs ≤ 5.6%. The only systematic difference was in the eumenorrheic group, where both absolute and relative fascicle lengthening was greater in visit 3 in comparison to visit 1. Similarly, only

eumenorrheic females produced a systematic difference for absolute and relative fascicle lengthening between-sets, despite very similar measures of reliability. One possible explanation for this is that, as the weakest group, as determined by PT force (Table 4-5), eumenorrheic females might be less trained than participants in the other group. As strength training reduces force fluctuations during maximal contractions (Tracy et al., 2004, González et al., 2007) and fascicle lengthening can be affected by contraction force (Fukunaga et al., 1997), the inability to maintain a consistent force at specific time points might account for the reported changes in fascicle lengthening. Nevertheless, the systematic differences in the eumenorrheic group were small (≤ 1.8%) and reliability measures remained high.

Conclusion

The present study can conclude that using US to measure PT and VL properties at rest and during maximal ECC exercise of the knee extensors is highly reliable and can be implemented when other imaging modalities are not available. Furthermore, there are minimal differences of the reliability of these measures between males, OCP users and eumenorrheic females. The overall aim of this thesis is to use 2D B-mode ultrasonography to further understand the properties and behaviour of the patella tendon and *vastus lateralis* in males and females and how these properties contribute to the EIMD response to ECC exercise and the magnitude of the RBE. The findings in the present study, and those from chapter three, are key to progressing to the next chapter. In chapter five, the US measures used in chapters three and four will be used to assess VL and PT properties at rest and during maximal knee extension exercise. Therefore, establishing the reliability of these US measures of MTU properties, along with understanding the measurement error relating to these techniques, is imperative to ensure that any changes that occur between the two exercise visits in chapter five results are not

misconstrued. An additional aim of this thesis is to compare the VL and PT behaviour during maximal ECC exercise and the magnitude of EIMD responses of males, OCP using females, and eumenorrheic females. The current study has established that the reliability of the US measures used to assess VL and PT behaviour during maximal ECC knee extension exercise does not differ between groups, thereby allowing comparisons between groups in chapter five to be completed with confidence.

CHAPTER 5 SEX DIFFERENCES IN EXERCISE INDUCED MUSCLE DAMAGE AND THE REPEATED BOUT EFFECT FOLLOWING MAXIMUM ECCENTRIC KNEE EXTENSION EXERCISE

5-1 INTRODUCTION

Humans produce an adaptive response to a single bout of maximal ECC exercise that reduces muscle damage markers in an identical bout performed a second time, known as the RBE (Nosaka and Clarkson, 1995). A number of mechanisms, including neural adaptations, remodelling of the ECM, changes in the inflammatory response and mechanical adaptations, such as a change in MTU behaviour, have been proposed to mediate the RBE, however these are not fully understood (Hyldahl et al., 2017, McHugh, 2003).

The mechanical behaviour of the MTU during contraction can be measured using ultrasonography. Chapter 4 established that US is a reliable, valid tool to measure MTU properties, specifically PT and VL properties at rest and during maximal isometric and lengthening exercise. Ultrasonography has previously been used to assess both tendon properties and muscle fascicle behaviour during iMVCs, passive movement, and a single bout of maximal eccentric contractions of the knee extensors, in both males and females (Finni et al., 2003, Guilhem et al., 2011, Hicks et al., 2013, Hicks et al., 2016). In recent years, US has been used to assess adaptations in the behaviour of the MTU of the VL during repeated bouts of ECC cycling (Penailillo et al., 2015) and MTJ displacement during repeated bouts of maximal ECC exercise of the elbow flexors (Lau et al., 2015). These studies found that men exhibit lower fascicle lengthening (Penailillo et al., 2015) and reduced muscle lengthening (Lau et al., 2015) on the repeated bouts of exercise. In a recent review of the mechanisms underpinning the RBE, Hyldahl et al. (2017) stated that this evidence of reduced muscle lengthening during a repeated bout of ECC exercise from Penailillo et al. (2015) and Lau et al (2015), suggests that there might be a possibility of an increase in tendon compliance, though direct evidence of this tendon adaptation has not been presented. Furthermore, the reduced amount of muscle lengthening displayed in the second bout of ECC exercise in the studies by

Penailillo et al. (2015) and Lau et al. (2015) were also accompanied by a lower magnitude of EIMD markers. Should the theory of tendon adaptation suggested by Hyldahl et al. (2017) be correct, this could explain the reduction in EIMD markers seen following a repeated bout of exercise in the studies by Penailillo et al. (2015) and Lau et al (2015) as a greater tendon compliance has been theorised to reduce the amount of fascicle lengthening during ECC exercise via a mechanical buffering action (Guilhem et al., 2016, Hicks et al., 2013). Therefore, it is possible that reduced muscle lengthening during a second bout of ECC exercise, via an increase in compliance of the tendon, could be a mechanism that explains the RBE. However, no study to date has measured tendon properties between two bouts of maximal ECC exercise, therefore it is unclear if this tendon adaptation exists.

The measurement of muscle lengthening during repeated bouts of ECC exercise has provided some insight into the role that MTU adaptations might play in reducing markers of EIMD (Lau et al., 2015, Penailillo et al., 2015), however, these studies only involved male participants and therefore do not take into account the differences in MTU behaviour that has been displayed in females. For example, in a single bout of maximal ECC exercise of the knee extensors, females have exhibited a lower magnitude of VL fascicle lengthening and higher PT compliance in comparison to males (Hicks et al. 2013, Hicks et al., 2016). As this observation is based on a single bout of ECC exercise, two pertinent questions require answering; 1) does the difference in MTU behaviour between males and females remain after repeated bouts of ECC exercise? and 2) does fascicle lengthening and the associated EIMD response reduce with the RBE, as seen in males (Penailillo et al., 2015)? Examining MTU behaviour and the EIMD response in males and females would give insight into whether MTU adaptations mediate the RBE, and whether these changes are consistent across sexes.

The baseline differences in the behaviour of MTU properties between males and females might be attributed to differences in sex hormones, specifically, oestrogen. For example, α and β oestrogen receptors are localised and expressed within tendon tissue and the increased action of these receptors in the presence of high circulating oestrogen levels might be responsible for the higher tendon compliance observed in females compared to males (Faryniarz et al., 2006, Hart et al., 1998, Wiik et al., 2003 Wiik et al., 2005, Sciore et al., 1998). This increased compliance, or lower tendon stiffness, is thought to be due to the chronic effects of oestrogen on collagenous tissue, which include decreased total collagen and protein content, tendon fascicle diameter and density (Belanger et al., 2004, Baltzopoulos, 1995, Abubaker et al., 1996), increased collagen degradation and decreased collagen synthesis (Neugarten et al., 2000, Fischer, 1973, Dyer et al., 1980), a reduction in tensile strength (Slauterbeck et al., 1999, Booth and Tipton, 1970), and an increase in elastic content (Shikata et al., 1979, Fischer and Swain, 1977). Due to these effects of oestrogen on tendon properties, it would be logical to suggest that lower levels of oestrogen would have the opposing effect, leading to higher tissue stiffness, which has indeed been evidenced by the lower compliance of tendons in males compared to eumenorrheic females (Hicks et al., 2013, Kubo et al., 2003). As lower levels of oestrogen can lead to differences in tendon properties (Kubo et al., 2003), it would make sense to hypothesise that supressing oestrogen, through such means as using the OCP (van Heusden and Fauser, 2002, Elliott-Sale et al., 2013), could result in decreased tendon stiffness. However, although OCP administration has been shown to reduce the stimulating effect of exercise on tendon collagen synthesis through the suppression of collagen fractional synthetic rate (Hansen et al., 2009a), differences in mechanical tendon properties, such as stiffness and Young's modulus, are not different between OCP users and eumenorrheic females (Hansen et al., 2013, Hicks et al., 2017). However, there is evidence to suggest that OCP use might augment the CK

response (Hicks et al., 2017, Joyce et al., 2014) and result in prolonged losses in iMVC strength (Minahan et al., 2015) in response to ECC exercise. Though far from conclusive, this evidence suggests that differences in the EIMD response to ECC exercise might differ between OCP users and eumenorrheic females. Moreover, this difference in EIMD response is only evident after a single bout of ECC exercise, so it remains unanswered if OCP users will respond differently to a repeated bout of ECC exercise compared to eumenorrheic females.

Chapters 3 and 4 of this thesis showed that US can be used to measure PT and VL properties at rest and during maximal exercise both accurately and reliably, over multiple visits. Chapter 4 showed specifically that the mechanical behaviour of the MTU did not differ during three visits over a 28-day time period, consisting of 10 maximal ECC contractions of the knee extensors per visit. This would suggest that if differences in MTU behaviour were present following a high-volume bout of maximal ECC exercise, these differences could be attributed to MTU adaptation, rather than measurement error. The aim of this study was to assess whether adaptations in MTU behaviour during maximal lengthening contractions could explain the RBE, and whether these adaptations differed between males, eumenorrheic females, and females taking the OCP. Moreover, an additional aim of this study was to investigate the potential relationships between MTU behaviour and the magnitude of EIMD and the RBE.

5-2 METHODS

5-2.1 Participants

A total of 36 participants were recruited for the study and divided into three groups (12 per group), males, OCP using females, and eumenorrheic females. Baseline characteristics are presented in Table 5-1. Participants completed a pre-test questionnaire and were included in the study if they had no neuromuscular or musculoskeletal impairments in the lower limbs

within the last six months. All participants self-reported being physically active, participating in no less than one lower body resistance training session per week. Females in the OCP group reported taking a combined monophasic OCP, with estradiol doses between 20-30 µg, for an average of 3 ± 2 years. The types of monophasic OCP used in this study were taken daily for 21 days, followed by a 7-day, withdrawal phase. Females in the eumenorrheic group reported a regular menstrual cycle (mean length 27 ± 3 days). A regular menstrual cycle was defined as 24-36 days (Landgren et al., 1980, Cole et al., 2009). Female participants with menstrual cycles outside of this regular range, or females who were pregnant in the year preceding the study were excluded. Institutional ethical approval was received from the Northumbria University Faculty of Health & Life Sciences Ethics committee in accordance with the *Declaration of Helsinki*. Participants were supplied with a participant information sheet, detailing the purpose of the study before giving written consent before participating (Appendix 1).

Table 5-1 Baseline participant characteristics.

	Male (n = 12)	OCP (n = 12)	Eumenorrheic (n = 12)	*P*
Age (years)	22 ± 4	23 ± 2	22 ± 3	0.557
Stature (m)	1.81 ± 0.06	1.71 ± 0.09	1.66 ± 0.06	0.002*#
Mass (kg)	83.9 ± 13.3	70.4 ± 15.0	64.3 ± 15.0	<0.001*#
VL CSA (cm^2)	34.0 ± 5.8	26.5 ± 5.2	28.2 ± 6.5	0.004*#
VL FL (mm)	69.6 ± 7.7	70.1 ± 14.2	62.3 ± 11.9	0.200
VL PA (degs)	19.4 ± 3.2	18.5 ± 2.2	20.2 ± 3.4	0.356
PTMA (cm)	4.3 ± 0.4	3.8 ± 0.4	3.8 ± 0.3	<0.001*#

Data are presented as mean ± SD. VL = vastus lateralis, CSA = cross-sectional area, FL = fascicle length, PA = pennation angle, PTMA = patella tendon moment arm, OCP = oral contraceptive pill females *P* = one-way ANOVA. * $P < 0.05$ males vs OCP; # $P < 0.05$ males vs eumenorrheic.

5-2.2 Procedures

Study design

Participants visited the laboratory on 13 occasions throughout the course of the study for one practice trial (single visit), followed by two experimental trials, each consisting of six visits. On the first day of the first experimental trial (referred to as Bout 1, or B1) participants completed baseline assessment of resting VL and PT properties, followed by assessment of baseline markers of EIMD, PT properties, and OA of force production during a concentric contraction. This was then followed by a bout of maximal ECC exercise. Follow up assessments of EIMD markers were performed at 24, 48, 72, 96 and 168 hours post-exercise. Approximately four weeks later, participants completed a second experimental trial that was identical to the first, to study the RBE referred to as Bout 2, or B2). Participants were instructed to refrain from exercise for 24 hours prior to baseline testing, and throughout the remainder of the seven-day testing period during both B1 and B2. Participants were instructed to refrain from the consumption of caffeine for 12 hours prior to testing, and to continue their habitual diet throughout the study. Participants were also instructed to avoid non-steroidal anti-inflammatory drugs or other stimulants for the duration of the experimental period. During the 4-week period between experimental trials participants were instructed to resume and maintain their habitual physical activity. For both muscle damage sessions, eumenorrheic females were tested during the mid-luteal phase of the menstrual cycle, seven days (± one day) after confirming ovulation, where both oestrogen and progesterone are high (Stricker et al., 2006). Follow up sessions were measured over the remainder of the luteal phase, into the early follicular phase, where oestrogen and progesterone gradually decline (Stricker et al., 2006). Female participants taking a monophasic OCP were tested on the 14th day (self-reported) of pill ingestion, equivalent to day 21 of the menstrual cycle, due to the initial seven pill-free days

(Elliott et al., 2005). This time-point is equivalent to the mid-luteal phase of eumenorrheic females taking no form of hormone influencing contraceptives.

Practice trial overview

The practice trial began with a DXA scan to measure the PTMA of the non-dominant leg (defined as the leg used to provide stability during movements e.g. kicking) at a 90° knee angle (0° = full extension). The participant was then permitted practice with the physical requirements of the testing procedure, which involved sitting in an isokinetic dynamometer (System 4 Pro, Biodex Medical Systems Inc., NY, USA) and taking the non-dominant leg passively through a pre-determined range of motion (30°-110° knee extension (full extension = 0°). The participant then performed iMVCs of the knee extensors against the lever arm of the dynamometer at 70° and 80° knee angles, followed by a ramped iMVC at 80°, with the aim of reaching maximum iMVC torque in a time of 4-6 seconds, as linear as possible. A knee angle of 80° was selected for the ramped iMVC in the practical trial to avoid isometric contractions at long muscle lengths, as this has been shown to confer a protective effect against markers of EIMD in subsequent damaging exercise (Chen et al. 2012b). The ramped iMVC in the practice trial was a manoeuvre that participants were required to execute during experimental visits to measure tendon stiffness and YM. Participants received a demonstration of maximal eccentric contractions of the knee extensors by the researcher, to habituate the participant with the action of the dynamometer. The participant did not perform any ECC knee extensor exercise in the practice trial, as previous research has demonstrated that even very small volumes of ECC exercise can confer a protective effect on subsequent bouts (Nosaka et al., 2001).

Experimental trials overview

A schematic of the experimental trials is illustrated in Figure 5-1. At baseline, anthropometric measurements (stature, mass, mid-thigh circumference (MTC) of the non-dominant leg) were recorded. B-mode ultrasound was then used to measure resting fascicle length and CSA of the VL, and length and CSA of the PT. Creatine kinase levels were measured via capillary blood sampling. Participants then completed ratings of muscle soreness, followed by isometric and concentric muscle actions of the knee extensors to measure PT stiffness, and to determine the optimum angle of force production respectively. Isometric MVCs of the knee flexors were performed to estimate co-activation force, which was used when calculating PT stiffness. Surface electromyography of hamstring muscle groups were recorded throughout measures of PT stiffness to measure co-activation (Onambele et al., 2007). Subsequent to baseline measures, participants then performed a bout of muscle damaging exercise consisting of 100 maximal isokinetic ECC knee extensions (20 sets of 5 repetitions, with one minute of rest between sets), during which fascicle lengthening was tracked at 50% of VL length and width using ultrasonography. Post exercise assessments of MTC, soreness, CK, iMVC and cMVC were measured after the ECC protocol at 24, 48, 72, 96 and 168 h (Figure 5-1). The experimental trials (B1 and B2) were identical.

Procedures for anthropometric measurements, PTMA, VL fascicle length and CSA, PT length and CSA, measures of iMVC and OA, measurements of VL fascicle lengthening during ECC exercise, ultrasound image analysis and confirmation of ovulation in eumenorrheic females were identical to the techniques used in Chapter 4; a detailed description can be found in section 4-2.3. The additional methods implemented in the current chapter are detailed below.

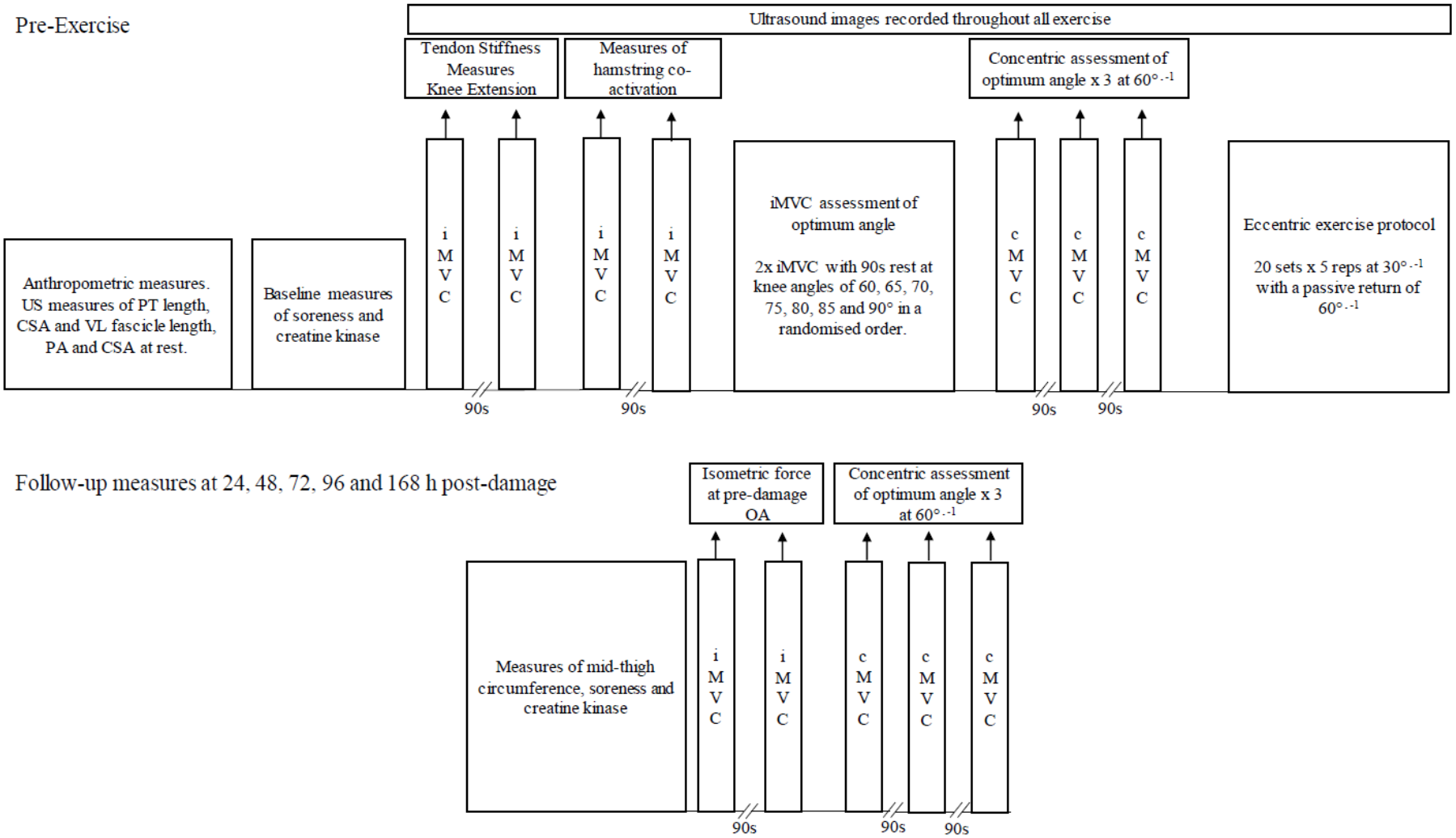

Figure 5-1 Schematic illustration of the experimental trial. The procedure was repeated ~4 weeks later. US = ultrasound; PT = patella tendon; CSA = cross-sectional area; VL = *vastus lateralis*; iMVC = maximal isometric voluntary contraction; cMVC maximal concentric voluntary contraction; OA = optimal angle.

Mid-thigh circumference

Mid-thigh circumference was measured using a standard anthropometric tape, at the point marked at 50% VL length and width as outlined in section 4-2.

Delayed onset of muscle soreness

Delayed onset of muscle soreness was quantified via VAS, which involved asking participants to mark on a scale of 0-200 mm (0 = no pain, 100 = very, very sore) their feeling of muscle soreness, when a 60 N pressure was applied at the same point marked for VL fascicle length analysis. The force applied was determined by a digital algometer (FDX 50 Force Ten digital force gauge, Wagner Instruments, CT, USA).

Creatine kinase

Fingertip capillary blood samples (20 μL) were taken using standard aseptic techniques. Samples were analysed using commercial CK analysis kit (Reflotron®, Roche Diagnostics, Risch-Rotkreuz, Switzerland), which has been shown to have excellent agreement with CK values obtained via venepuncture (Knoblauch et al., 2010). The resting normal expected values for CK when using this equipment are between 50 and 200 $IU \cdot L^{-1}$; the CV for this instrument was < 3% (Keane et al., 2015).

Measures of concentric force and optimal angle

Maximal concentric voluntary contraction (cMVC) torque (N·m) was assessed during isokinetic contractions performed at $60° \cdot s^{-1}$ angular velocity from 110-30° knee angle. Three efforts were performed with one-minute rest between repetitions. The repetition with the highest peak torque was used for further analysis, with the knee angle at which peak torque

occurred being recorded as the OA of force production. This procedure has been shown to be reliable and suitable for measuring OA in the knee extensors (Oranchuk et al., 2020). This procedure was repeated in all follow up sessions after both eccentric exercise protocols to monitor any changes in the length-tension relationship. Changes in OA have been shown following damaging bouts of exercise and the use of cMVC has been shown to be sensitive in detecting changes in OA (Chen et al., 2007, Chen et al., 2013).

Patella tendon stiffness and Young's modulus

A detailed description of the method used to calculate PT stiffness and YM and the methods used to measure surface EMG and antagonist co-activation during ramped iMVC, can be found in section 4-2.3. In the current study, both PT stiffness and YM were standardised using two methods, to allow specific comparisons. For each participant, the slope of the tangential line was computed for the weakest PT force recorded for each individual participant, across the two bouts and used to calculate PT stiffness and YM. This is referred to as "within participant" stiffness or YM and is used to allow the comparison of PT properties at an individual level. This standardised the comparison of PT stiffness and YM at a consistent load within each participant, across bouts. Additionally, for each participant, the slope of the tangential line was computed for the weakest PT force recorded by all participants, across the two bouts and used to calculate PT stiffness and YM. This is referred to as "between participant" stiffness or YM. This standardised the comparison of PT stiffness and YM at a consistent load between each participant (Onambele et al., 2007) and is used to assess differences in PT properties between participants, irrespective of strength.

Index of protection

To quantify the magnitude of the protective effect between bouts, the IOP was calculated for MTC, DOMS, CK, iMVC and cMVC. Index of protection was calculated by the following equation [(B1 – B2) / B1 × 100] (Chen et al. 2016). The timepoint compared for each variable between bouts was the point of maximal muscle damage response following the first damaging bout. This was individualised for each participant to account for EIMD time course variability between participants.

Eccentric exercise protocol

The muscle damage protocol consisted of 20 sets of 5 maximal ECC actions of the knee extensors in the isokinetic dynamometer, at an angular velocity of $30°\cdot s^{-1}$ during the eccentric phase, and a passive return to full extension at $60°\cdot s^{-1}$ in the concentric phase, with one minute between sets. Maximal voluntary eccentric knee extension torque was recorded during each action and displayed via the torque acquisition system. Real-time feedback was displayed to the participants throughout the exercise protocol, along with strong verbal feedback, to ensure that maximum effort was performed during each repetition.

VL fascicle lengthening

Image acquisition and analysis of fascicle lengthening has been previously described in section 4-2.3. To quantify VL fascicle lengthening during the ECC exercise protocol, two repetitions were selected for analysis. One at the point at which the participant was 'fresh' (set 1, rep 5) and the other when it was expected that force decrements would occur (set 20, rep 5). Images were analysed offline (at every 10° during eccentric contractions) using imaging software (ImageJ 1.45; National Institutes of Health, Bethesda, MD, USA). Fascicle lengths from the

still images were analysed as previously described (Figure 4-2). Fascicle length changes were measured as absolute change (FL at 110° knee angle – FL at 30° knee angle) and relative change (percentage increase in FL from 30° (0%), in 10° increments, to a knee angle of 110°).

5-2.3 Statistical analysis

Statistical analysis was performed using SPSS (v20, SPSS Inc., Chicago, IL). All data are reported as mean ± SD. CK data was log transformed prior to analysis due to data not being normally distributed. Sphericity was assessed using Mauchly's test of sphericity. Where sphericity was violated, a greenhouse-Geisser correction was used (Maxwell and Delaney, 2004). To investigate the effect of the RBE independent of group, data for all participants were combined and responses to different bouts over time were assessed using a 2 × 6 ANOVA (bout × time). To investigate whether responses differed between males, eumenorrheic females, and OCP-using females, differences in responses between groups across bouts (B1 and B2) over time (pre, 24, 48, 72, 96 and 168 hours) were assessed using a 3 × 2 × 6 ANOVA (group × bout × time). Where significant group × bout × time interactions were present, a series of two-way ANOVAs were performed to compare between groups for the first bout and second bout. Where significant bout × time interactions were present, least significant difference *post hoc* comparisons were implemented to assess differences between bouts. Where data were combined, paired sample t-tests were used to assess for differences at baseline between bouts. One-way ANOVAs were used to assess for differences in IOP between groups. Where a significant difference was present, least significant difference *post hoc* comparisons were implemented to assess differences in IOP between groups. Paired sample t-tests were used to assess for differences in total work (kJ) between bouts. A 3 × 2 ANOVA (group × bout) was used to assess for differences between groups at baseline. Where significant group × bout interactions were present, least significant difference *post hoc* comparisons were implemented

to assess differences between bouts. Linear correlations (Pearson *r*) were used to investigate whether significant associations were present between markers of EIMD, VL MTU behaviour and IOP. Statistical significance was set at an alpha level of 0.05.

5-5 RESULTS

Baseline characteristics

Participant baseline characteristics are presented in Table 5-1. There were no between group differences in age ($P = 0.557$), VL FL ($P = 0.200$) or PA ($P = 0.356$). Males were taller than OCP using females (+0.10 m, $P < 0.001$) and eumenorrheic females (+0.15 m, $P < 0.001$) with no differences between OCP using females and eumenorrheic females ($P = 0.127$). Males were heavier than OCP using females (+13.5 kg, $P = 0.015$) and eumenorrheic females (+19.6 kg, $P < 0.001$), with no differences between OCP using females and eumenorrheic females ($P = 0.257$). Males had a larger VL CSA than the OCP group (+7.5 cm^2, $P = 0.001$) and eumenorrheic group (+5.8 cm^2, $P < 0.001$), with no differences between OCP using females and eumenorrheic females ($P = 0.228$). Males had a longer PTMA than the OCP using females (+0.5 cm, $P = 0.004$) and eumenorrheic females (+0.5cm^2, $P < 0.003$), with no differences between OCP using females and eumenorrheic females ($P = 0.883$).

Sex hormone concentrations in eumenorrheic females

Serum estradiol levels were similar between B1 (146.3 ± 51.4 $pg \cdot ml^{-1}$) and B2 (148 ± 64.7 $pg \cdot ml^{-1}$, $P = 0.414$). Serum progesterone levels were similar between B1 (12.1 ± 5.1 $ng \cdot ml^{-1}$) and B2 (11.8 ± 5.7 $ng \cdot ml^{-1}$, $P = 0.271$), confirming, in addition to positive ovulation tests, that eumenorrheic females were ovulatory and in the mid-luteal (progesterone > 7.9 $ng \cdot ml^{-1}$) phase of the menstrual at the time of testing in both visits (Stricker et al., 2006, Arce et al., 2011).

Eccentric exercise and markers of EIMD

The total amount of work performed by all participants was similar for B1 and bout B2 (19.6 ± 4.8 kJ and 19.9 ± 4.6 kJ, respectively. $P = 0.198$). Males performed more total work than the OCP and eumenorrheic group in B1 (24.3 ± 2.7 kJ vs 17.6 ± 3.8 kJ, $P < 0.001$ and 16.8 kJ $P < 0.001$) and B2 (24.1 ± 3.1 kJ vs 18.3 ± 3.7 kJ, $P < 0.001$ and 17.2 kJ $P < 0.001$), with no difference between OCP users and eumenorrheic female groups in B1 or B2 ($P = 0.577$ and $P = 0.464$, respectively).

Isometric strength

Changes in iMVC relative to baseline following ECC exercise are displayed in Figure 5-2 (a) for B1 and Figure 5-2 (b) for B2. In B1, iMVC force loss peaked at 24 h (77 ±14% of pre ECC, $P < 0.001$) and remained below baseline at 168 h (95 ± 12% of pre ECC, $P = 0.013$). In B2, iMVC reduction peaked at 24 h (92 ± 13% of pre ECC, $P < 0.001$) and returned to baseline by 48 h. iMVC force loss was lower in B2 compared to B1 based on a main effect of bout ($P < 0.001$), and at all time points ($P < 0.001$), based on a bout × time interaction ($P < 0.001$). No group differences were present (bout × time × group $P = 0.112$).

Concentric strength

Changes in cMVC relative to baseline following ECC exercise are displayed in Figure 5-2 (c) for B1 and Figure 5-2 (d) for B2. In B1, cMVC force loss peaked at 24 h (80 ± 19% of pre ECC, $P < 0.001$) and returned to pre- ECC levels by 168 h. In B2, cMVC reduction peaked at 24 h (89 ± 13% of pre ECC, $P < 0.001$) and returned to baseline by 72 h. cMVC force loss was lower in B2 compare2 to B1 based on a main effect of bout ($P < 0.001$). cMVC force loss was lower in B2 compared to B1 at 24 h (–8.5% $P = 0.014$), 48 h (–8.9%, $P = 0.007$), 72 h (–9.2%,

P = 0.006) and at 96 h (–10.3%, P = 0.005), based on a bout × time interaction (P = 0.012). No group differences were present.

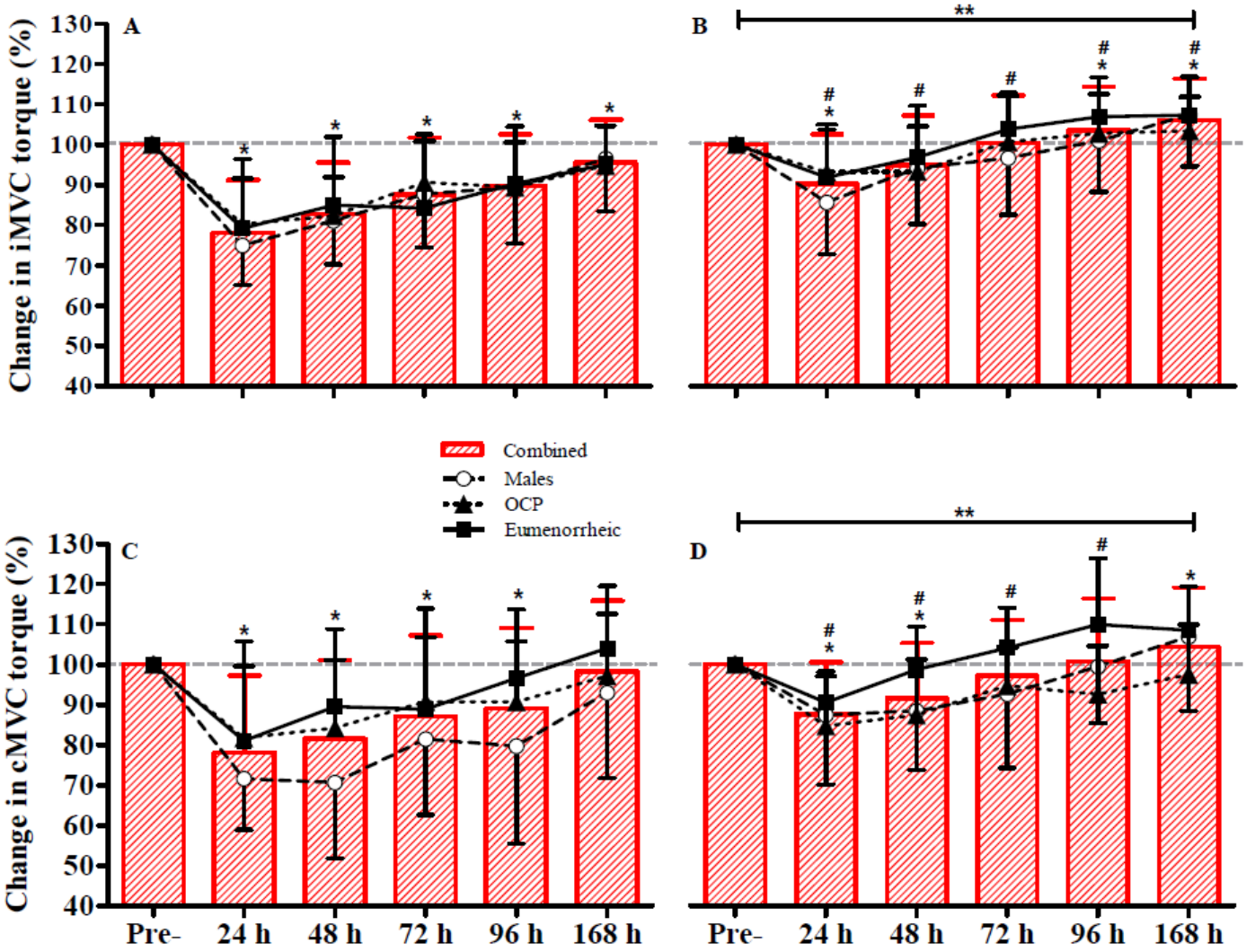

Figure 5-2 Changes in maximal isometric force following ECC exercise in B1 (a) and bout 2 (b) and changes in maximal concentric force following ECC exercise in B1 (c) and B2 (d). iMVC = maximal isometric voluntary contraction, cMVC maximal concentric voluntary contraction, OCP = oral contraceptive pill, * P = < 0.05 compared to pre-exercise levels within bout; ** P < 0.05 bout 1 vs bout 2; # P < 0.05 bout 1 vs bout 2 at the respective time point. The grey dashed line represents 100% of pre-exercise values. Combined n = 36, males, n =12; OCP females n = 12, eumenorrheic females, n = 12.

Delayed onset of muscle soreness

Changes in DOMS are displayed in Figure 5-3 (a) for B1 and Figure 5-3 (b) for B2. DOMS peaked at 48 h post ECC in B1 and B2 (106.0 ± 40.6 mm and 85.9 ± 48.7 mm, respectively) and had returned to the respective pre-ECC levels by 168 h in both bouts. DOMS was lower in B2 in comparison to B1, based on a main effect of bout ($P < 0.001$) but no bout × time interaction was present ($P = 0.158$). No further interactions or group differences were present.

Creatine Kinase

Changes in CK are displayed in Figure 5-3 (c) for B1 and Figure 5-3 (d) for B2. In B1, CK levels were increased from pre ECC levels by 312.4 IU·L^{-1} at 24 h ($P < 0.001$), with a maximum absolute increase of 1331.3 IU·L^{-1} at 96 h ($P < 0.001$) and remained elevated at 168 h. In B2, CK levels were increased from pre ECC levels by 157.1 IU·L^{-1} at 24 h ($P < 0.001$), reduced but remained elevated by 63.7 IU·L^{-1} at 48 h ($P = 0.011$), before returning to pre ECC levels by 72 h. CK levels were lower in B2 compared to B1 based on a main effect of bout ($P < 0.001$). There were no differences between pre ECC CK levels in B1 and B2 ($P = 0.882$). CK levels were lower in B2 in comparison to B1 at 24 h (–147.5 IU·L^{-1}, $P = 0.005$), 48 h (–435.4 IU·L^{-1}), $P < 0.001$), 72 h (–522.7 IU·L^{-1}, $P = 0.001$), 96 h (–1298.4 IU·L^{-1}, $P < 0.001$), and 168 h (–672.6 IU·L^{-1}, $P < 0.001$). No group interactions were present. To account for differences in VL CSA, between the male group and the OCP using and eumenorrheic female groups, CK was normalised to VL CSA and re-analysed. This analysis revealed no differences between groups.

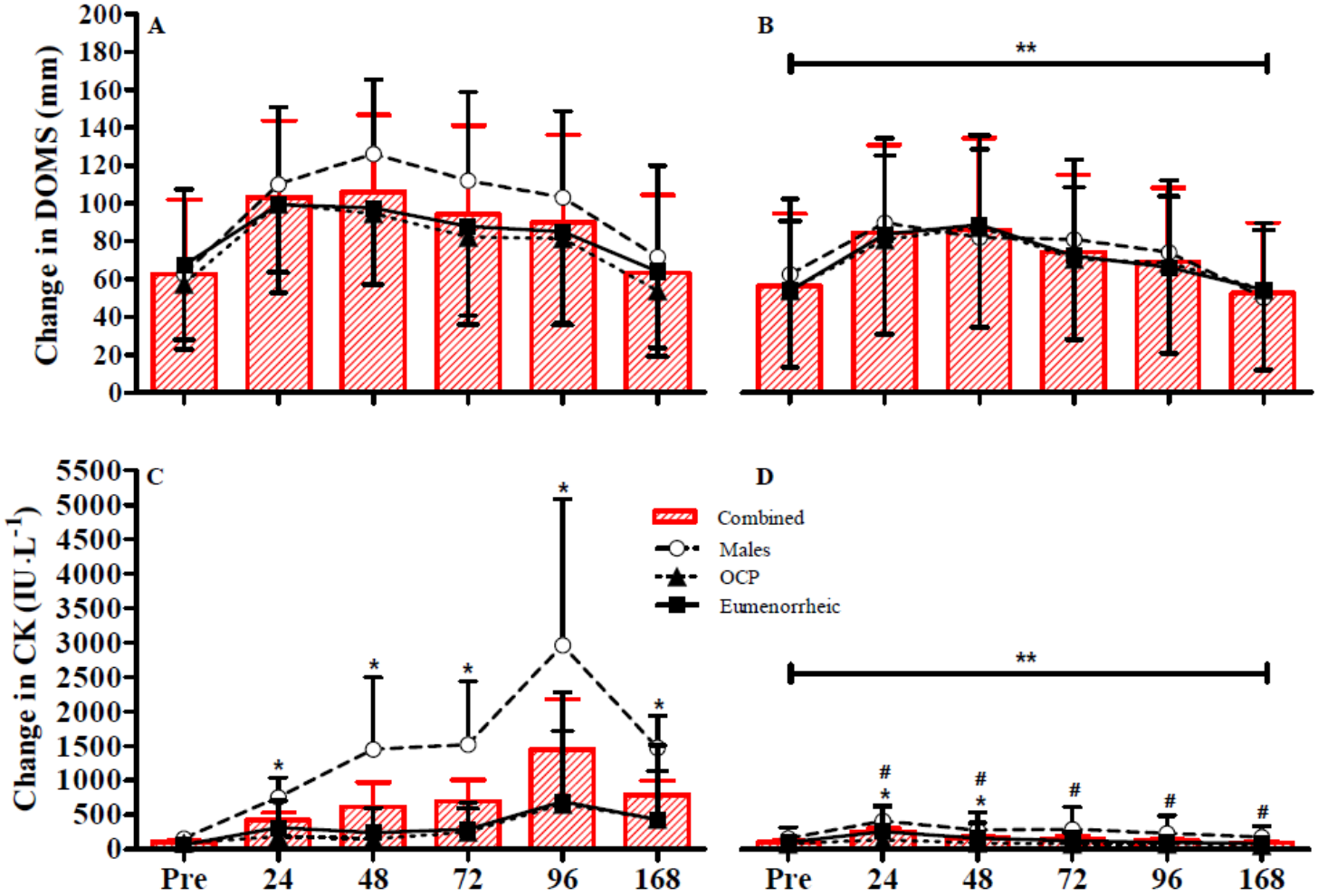

Figure 5-3 Changes in delayed onset of muscle soreness (DOMS) following ECC exercise in B1 (a) and bout 2 (b) and changes in CK following ECC exercise in B1 (c) and B2 (d). DOMS = delayed onset of muscle soreness, CK = creatine kinase, OCP = oral contraceptive pill, * $P < 0.05$ compared to pre-exercise levels within bout for all participants combined; ** $P < 0.05$ bout 1 vs bout 2 for all participants combined; # $P < 0.05$ bout 1 vs bout 2 at the respective time point for all participants combined. Combined n = 36, males, n =12; OCP females n = 12, eumenorrheic females, n = 12.

Optimal angle of force production from concentric knee extension

Changes in OA are displayed in Figure 5-4a for B1, and Figure 5-4b for B2. A main effect of bout ($P = 0.021$) showed that OA of force production from concentric knee extension was at a longer muscle length in B2 in comparison to B1 (69.5 ± 7.9 vs 67.7 ± 9.0°, respectively, $P = 0.021$). No other interactions including group or time were present.

Mid-thigh circumference

Changes in MTC are displayed in Figure 5-4c for B1, and Figure 5-4d for B2. In B1, the largest increase on MTC was recorded at 48 h (+1.2% vs pre ECC, $P < 0.001$) and returned to pre ECC levels by 168 h. In B2, MTC peaked at 24 h (+0.7% vs pre ECC, $P < 0.001$) and returned to pre-ECC levels by 96 h. A main effect of bout showed that overall MTC was lower in B2 in comparison to B1 (100.3 ± 0.7% vs 100.6 ± 1.0%, respectively, $P = 0.006$). There was a bout × time interaction, with post-hoc tests showing differences between B1 and B2 at 48 h (101.2 ± 1.0% vs 100.6 ± 0.8%, respectively, $P = 0.003$), 72 h (100.9 ± 0.7% vs 100.3 ± 0.7%, respectively, $P = 0.001$) and 96 h (100.5 ± 0.7% vs 100.1 ± 0.7%, respectively $P = 0.027$). No between group differences were present.

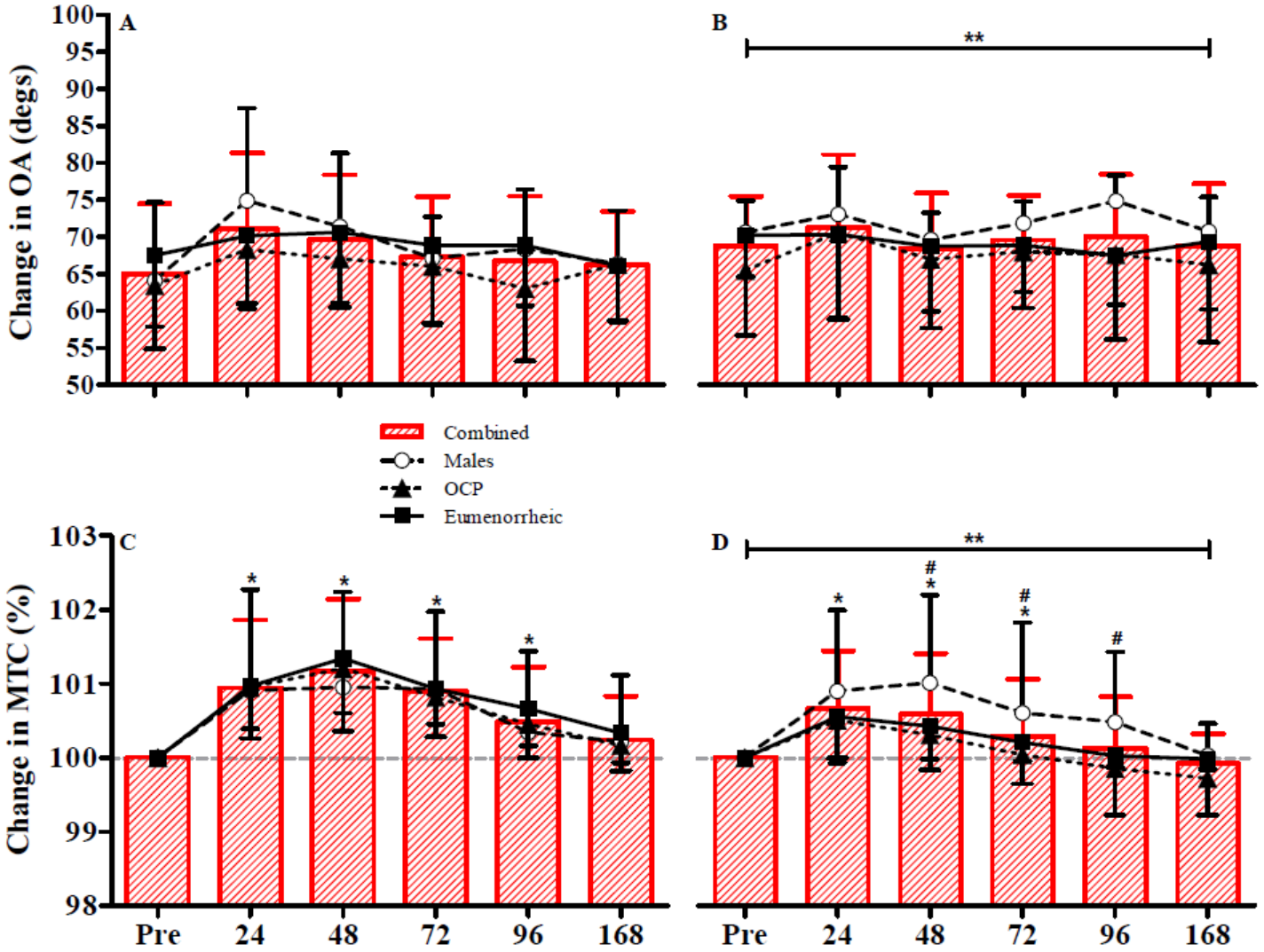

Figure 5-4 Changes in OA following ECC exercise in B1 (a) and bout 2 (b) and changes in MTC following ECC exercise in B1 (c) and B2 (d). OA = Optimal angle of force production, MTC = mid-thigh circumference, OCP = oral contraceptive pill, * P = < 0.05 compared to pre-exercise levels within bout; ** P < 0.05 bout 1 vs bout 2; # P < 0.05 bout 1 vs bout 2 at the respective time point. The grey dashed line represents 100% of pre-exercise values. Combined n = 36, males, n =12; OCP females n = 12, eumenorrheic females, n = 12.

Patella tendon properties prior to ECC exercise in bouts 1 and 2

For combined data, PT CSA was similar between both bouts (Table 5-2). Patella tendon stiffness for within-participant (standardised to the lowest tendon force across both bouts, per participant) and between-participant (standardised to the weakest tendon force for all participants, 1612 N) was not statistically different between bouts (P = 0.088 and P = 0.089, respectively). Between participant YM was lower in B2 in comparison to B1 (597.8 ± 134.0 MPa vs 629.8 ± 163.8 MPa, respectively, P = 0.05). Within participant YM was not statistically different between bouts (P = 0.058). Males displayed higher within-participant stiffness in B1 (1405.7 ± 449.5 N·mm^{-1}), in comparison to the OCP group (1045.1 ± 264.5 N·mm^{-1}, P = 0.013) and eumenorrheic group (958.2 ± 280.8 N·mm^{-1}, P = 0.003), but not in B2 (P = 0.051). No other differences in PT stiffness or YM between groups were present (Figure 5-5).

Table 5-2 Pre-ECC exercise VL and patella tendon properties and optimal angle of force production for all participants combined (n = 36).

	Bout 1	Bout 2	*P*
VL CSA (cm^2)	28.2 ± 6.5	28.2 ± 6.4	0.175
VL FL (mm)	67.3 ± 11.9	65.0 ± 15.4	0.196
VL PA (degs)	19.4 ± 3.0	19.2 ± 3.0	0.069
PT CSA (mm^2)	71.1 ± 14.7	70.5 ± 13.4	0.435
PT Stiffness			
Within (N·mm^{-1})	1136.3 ± 385.9	1087.4 ± 353.4	0.088
Between (N·mm^{-1})	758.4 ± 174.5	729.2 ± 139.2	0.089
PT Young's Modulus			
Within (MPa)	925.7 ± 265.1	875.8 ± 244.9	0.058
Between (MPa)	629.8 ± 163.8	597.8 ± 134.0	0.05*
Optimal Angle (degs)			
iMVC	72.2 ± 6.9	73.9 ± 6.2	0.070
cMVC	65.0 ± 9.5	68.8 ± 6.7	0.005*

Data are presented as mean ± SD. VL = *vastus lateralis*, PT = patella tendon, CSA = cross-sectional area, FL = fascicle length, PA = pennation angle, iMVC = maximal isometric voluntary contraction, cMVC = maximal concentric voluntary contraction, P = paired sample t-test.

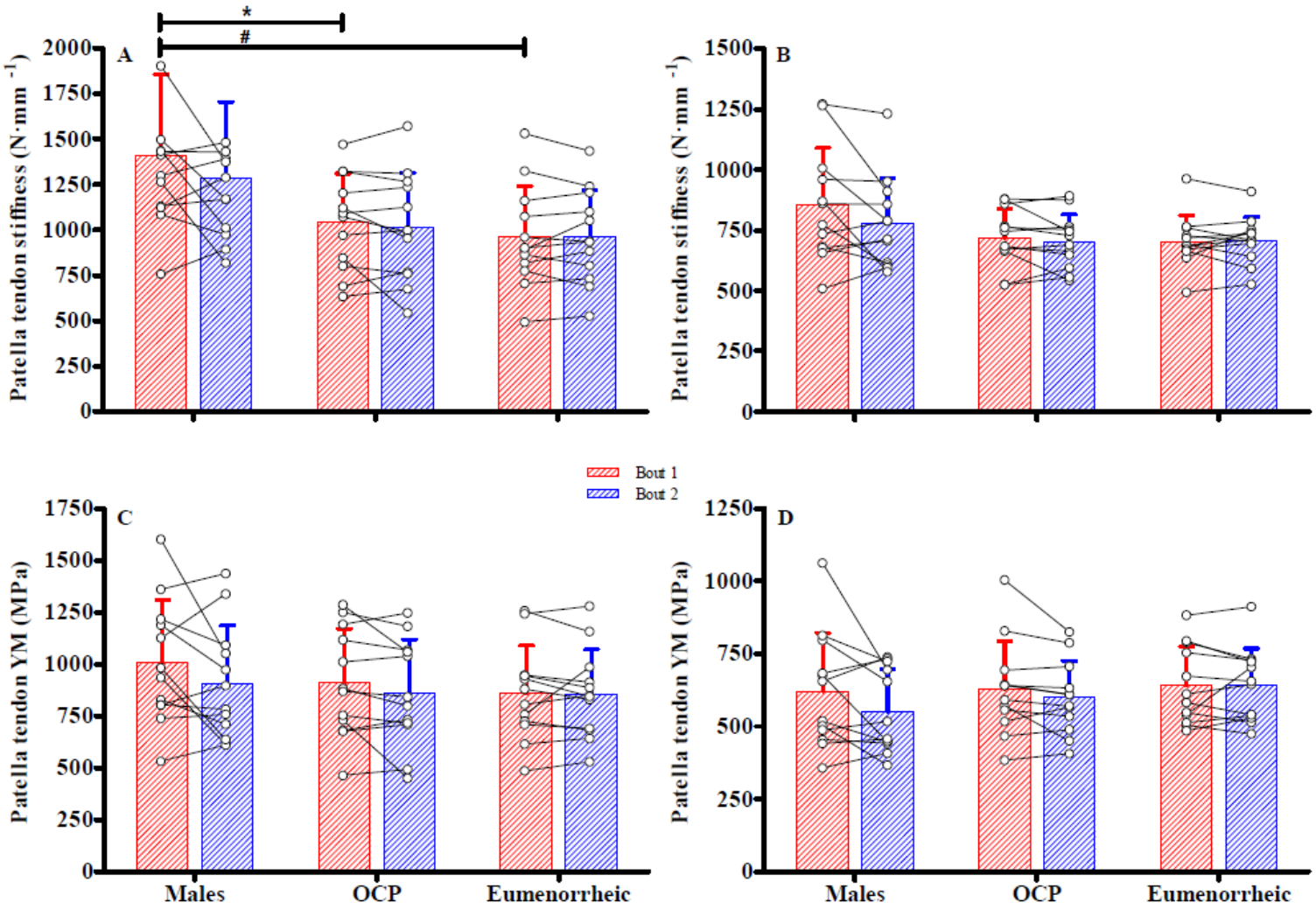

Figure 5-5 (A) Pre-exercise patella tendon stiffness values for each group relative to the lowest patella tendon force within participant, across the two bouts. (B) Pre-exercise patella tendon stiffness for each group relative to the lowest patella tendon force produced across the two bouts. (C) Pre-exercise patella tendon YM scores for each group relative to the lowest patella tendon force within participant, across the two bouts. (D) Pre-exercise patella tendon YM scores for each group to the lowest patella tendon force produced across the two bouts. YM = Young's modulus; OCP = oral contraceptive pill; * $P < 0.05$ males vs OCP using females; # $P < 0.05$ males vs eumenorrheic females. Males, n =12; OCP females n = 12, eumenorrheic females, n = 12.

Vastus lateralis properties prior to ECC exercise in bouts 1 and 2

There were no changes in VL CSA, FL, or PA between B1 and B2 (Table 5-2). Additionally, there was no difference in OA derived from iMVC between B1 and B2 (Table 5-2).

Vastus lateralis fascicle behaviour during ECC exercise

For all participants combined, absolute and relative VL fascicle lengthening during set 1 of ECC exercise was reduced in B2 in comparison to B1 by 4.8 mm and 7.5% respectively (both $P < 0.001$, Table 5-3). Absolute and relative VL fascicle lengthening during set 20 of ECC exercise was reduced in B2 in comparison to B1 by 5.3 mm and 7.7% respectively (both $P < 0.001$), respectively (Table 5-3). Vastus lateralis fascicle lengthening during ECC exercise reduced similarly between B1 and B2 for all groups, with no group differences in VL FL behaviour (Figure 5-6).

Table 5-3 VL fascicle lengthening during ECC exercise for all participants (n = 36).

	Bout 1	Bout 2	*P*
Set 1			
Absolute FL (mm)	45.4 ± 9.0	40.6 ± 7.5	<0.001*
Relative FL (%)	66.7 ± 18.2	59.2 ± 14.8	<0.001*
Set 20			
Absolute FL mm)	46.7 ± 9.1	41.4 ± 8.2	<0.001*
Relative FL (%)	65.1 ± 15.5	57.4 ± 13.4	<0.001*

Data are presented as mean ± SD. VL = vastus lateralis, FL = fascicle lengthening, *P* = paired sample t-test.

Set 1 fascicle lengthening by knee angle

Combined data for ECC torque and VL fascicle lengthening at every 10° throughout the full range of motion (30-110°) are displayed in Figure 5-7, for both bouts. Fascicle lengthening between B1 and B2 during set 1 remained similar at lower knee angles (between 30-70° for absolute FL, and 30-60° for relative FL). At higher knee angles, fascicle lengthening was reduced in B2 vs B1. Specifically, for relative fascicle lengthening, B2 showed reduced fascicle lengthening in comparison to B1 at a knee angle of 70° (–3.1%, $P = 0.017$), and for both absolute and relative scores, fascicle lengthening was reduced at knee angles between 80° and 110° (80° absolute –1.8 mm, $P = 0.045$, relative –4.0%, $P = 0.010$; 90° absolute –1.7 mm, $P = 0.034$, relative –3.8%, $P = 0.008$; 100° absolute –3.5 mm, $P < 0.001$, relative –6.5%, $P < 0.001$; and 110° –4.3 mm, $P < 0.001$, relative –7.5%, $P < 0.001$).

Set 20 fascicle lengthening by knee angle

Similar to set 1, absolute and relative VL fascicle lengthening between B1 and B2 during set 20 remained similar at lower knee angles between 30-60°. B2 showed reduced fascicle lengthening in comparison to B1 at knee angles of 70° (absolute –2.3 mm, $P = 0.049$, relative –3.3%, $P = 0.033$), 80° (absolute –2.7 mm, $P = 0.016$, relative –4.1%, $P = 0.014$), 90° (absolute –3.7 mm, $P = 0.001$, relative –5.6%, $P = 0.001$), 100° (absolute –4.9 mm, $P < 0.001$, relative –7.5%, $P < 0.001$) and 110° (absolute –5.1 mm, $P < 0.001$, relative –7.7%, $P < 0.001$).

Set 1 eccentric torque by knee angle

Total ECC torque across all knee angles was similar in B1 and B2, based on no main effect of bout ($P = 0.096$). There was a bout × time interaction ($P = 0.048$), with *post hoc* analysis revealing that ECC torque was higher in B2 in comparison to B1 at knee angles of 70° (+20.8

N·m, $P = 0.003$) and 80° (+26.7 N·m, $P = 0.001$). ECC torque was similar between groups based on no bout × angle × group interaction ($P = 0.539$) and no bout × group interaction ($P = 0.902$).

Set 20 eccentric torque by knee angle. Total ECC torque was similar between B1 and B2 in set 20, based on no main effect of bout ($P = 0.437$) with no other interaction effects present. Eccentric torque was similar between groups based on no bout × angle × group interaction ($P = 0.186$) and no bout × group interaction ($P = 0.163$).

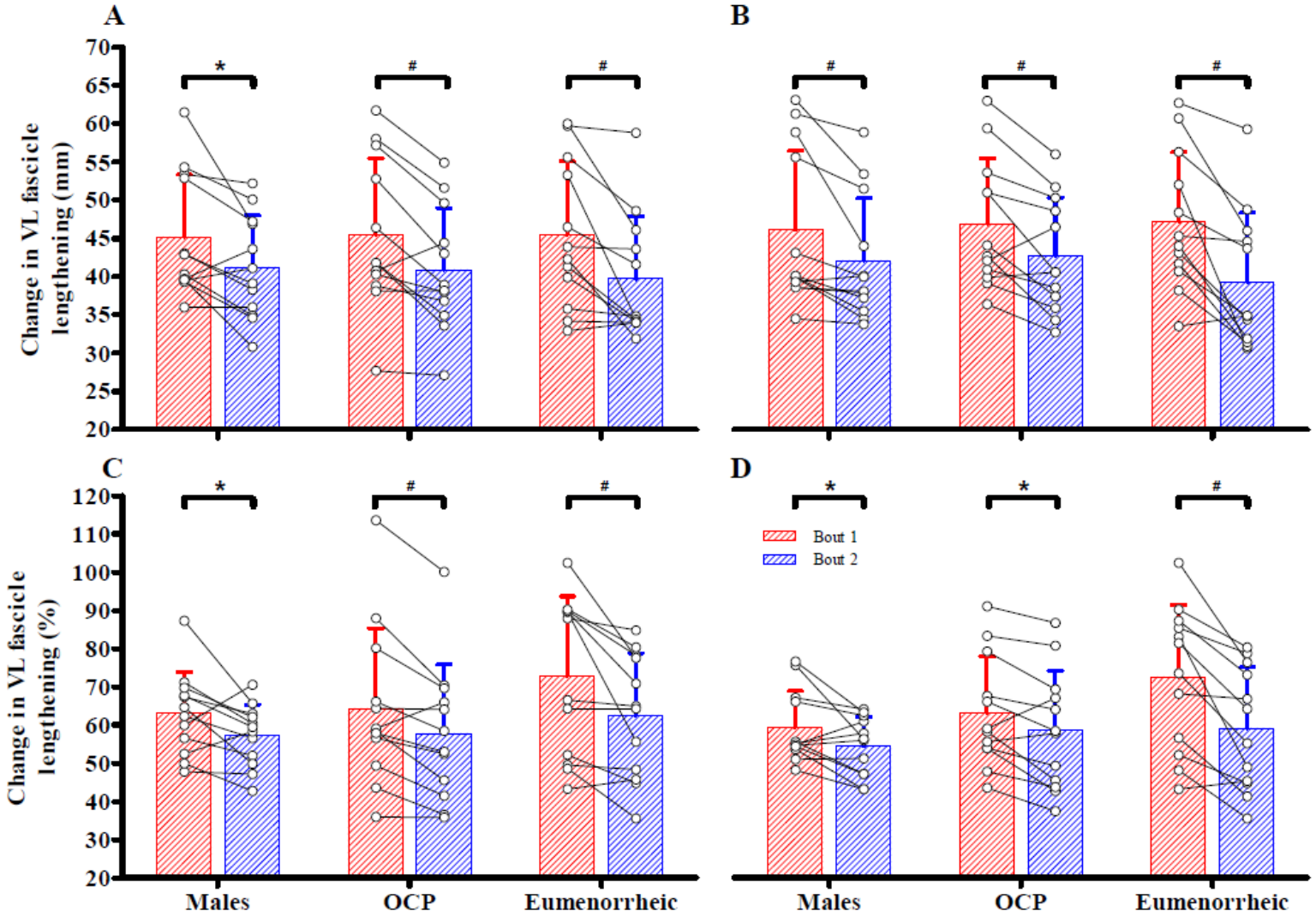

Figure 5-6 Absolute change in VL fascicle length for each group during set 1 (a) and set 20 (b) of eccentric exercise. Relative change in VL fascicle length for each group during set 1 (c) and set 20 (d) of eccentric exercise. VL = *vastus lateralis*, OCP = oral contraceptive pill, * $P < 0.01$ bout 1 vs bout 2; # $P < 0.001$ bout 1 vs bout 2. Males, n =12; OCP females n = 12, eumenorrheic females, n = 12.

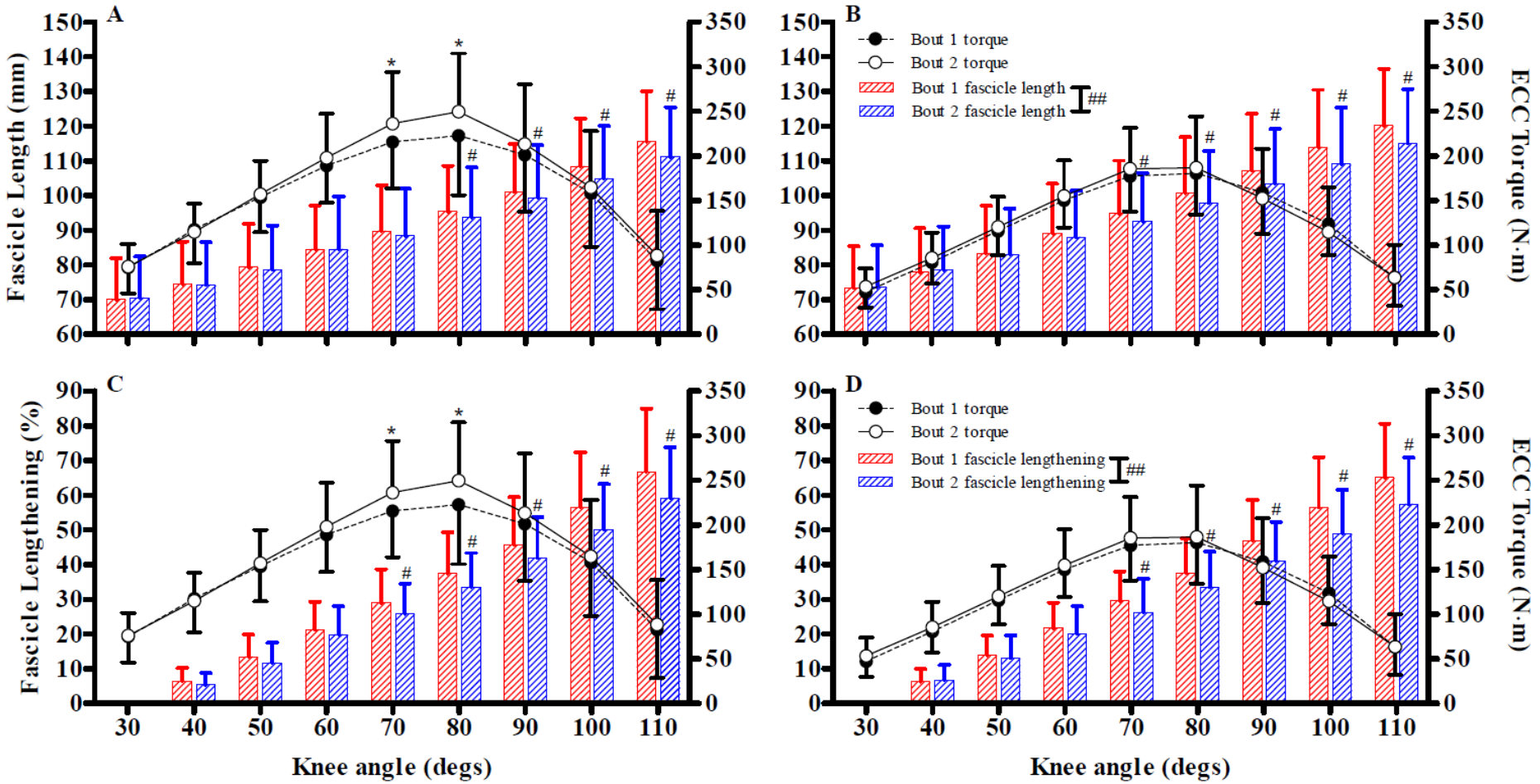

Figure 5-7 VL fascicle length (left y axis) and ECC torque (right y axis) every 10° knee angle during set 1 (a) and set 20 (b) of ECC exercise. Percentage change in VL fascicle length (left y axis) and ECC torque (right y axis) every 10° knee angle during set 1 (c) and set 20 (d) of ECC exercise. ECC = eccentric; * $P < 0.05$ B1 vs B2 torque at the respective time point; # $P < 0.05$ B1 vs B2 at the respective time point; ## $P < 0.01$ B1 vs B2. n = 36.

Index of protection

Figure 5-8 shows the index of protection for each marker of EIMD. MTC was omitted from Figure 5-8 due to the low values, which are presented below. With all participants combined, the largest protective effect was present for CK (54 ± 43%), followed by cMVC (28 ± 27%), DOMS (26 ± 35%), iMVC (24 ± 20%) and MTC (0.7 ± 1.2%). For cMVC, males had a higher IOP than the OCP group (43 ± 24% vs 14 ± 21%, $P = 0.006$). No other group differences were present.

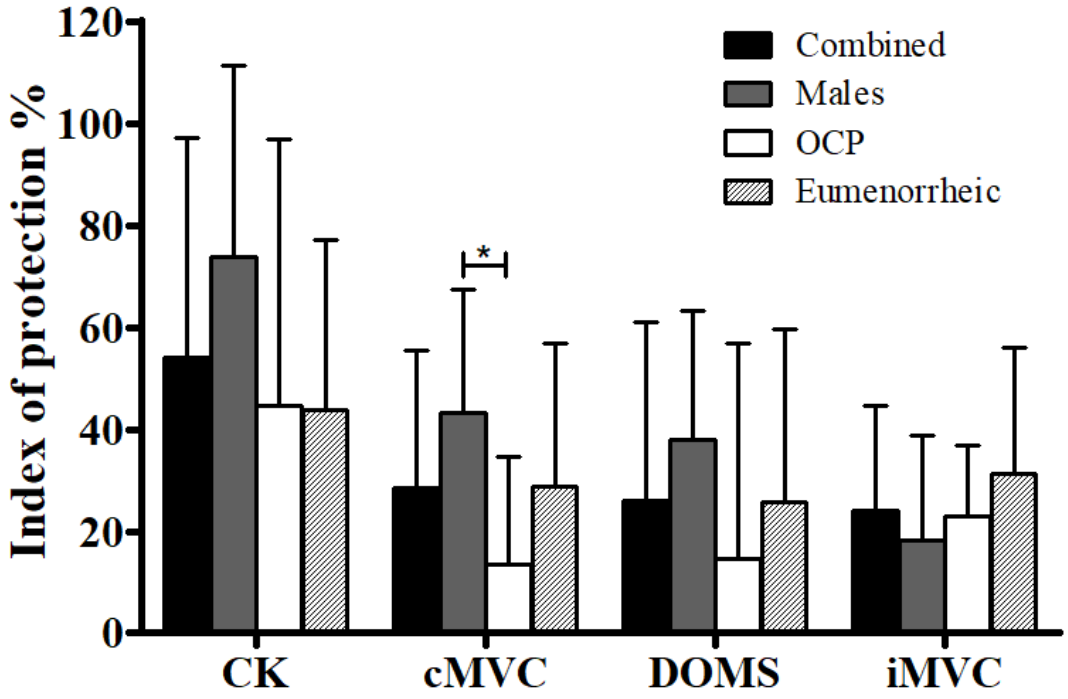

Figure 5-8 Index of protective effect for creatine kinase (CK), maximal concentric strength (cMVC), delayed onset of muscle soreness (DOMS) and maximal isometric strength (iMVC). OCP = oral contraceptive pill. * $P = 0.006$ males vs OCP using females. Index of protection was calculated by the following equation [(B1 - B2) / B1 × 100]. The timepoint compared for each variable used was the point of maximal muscle damage response following B1 for each individual. Combined n = 36, males, n =12; OCP females n = 12, eumenorrheic females, n = 12.

Associations between fascicle lengthening and markers of EIMD and the RBE

In bout one, relative VL fascicle lengthening was significantly correlated with absolute and relative losses in iMVC force at 24 h post exercise (r = –0.51, P = 0.002 and r = –0.39, P = 0.020, respectively), cMVC force at 24 h post exercise (r = –0.36, P = 0.030 and r = –0.43, P = 0.010, respectively) in B1. No other significant correlations were found between VL fascicle lengthening and markers of EIMD in either bout.

A greater IOP for iMVC was significantly correlated with greater absolute (Figure 5-9a, r = 0.44, P = 0.007) and relative (Figure 5-9b, r = 0.34, P = 0.044) magnitude of VL fascicle lengthening during set 1 of eccentric exercise. A greater IOP for iMVC was significantly correlated with greater absolute (Figure 5-9c, r = 0.43, P = 0.008) and relative (Figure 5-9d, r = 0.37, P = 0.026) VL fascicle lengthening during set 20 of eccentric exercise. The IOP for iMVC was also significantly correlated with the amount of absolute (Figure 5-10a, r = 0.41, P = 0.013) and relative (Figure 5-10b, r = 0.52, P = 0.001) iMVC force loss at 24 h post-exercise in B1. However, it must be acknowledged that the r^2 values for all relationships were low (≤ 0.27), therefore must be interpreted with caution. No other significant correlations between VL MTU properties and EIMD markers or IOP were present. There was no correlation between the reduction in VL fascicle lengthening between bouts and the IOP. Additionally, in the eumenorrheic female group, serum estradiol levels were not significantly correlated with VL MTU properties, EIMD markers following ECC exercise, or IOP.

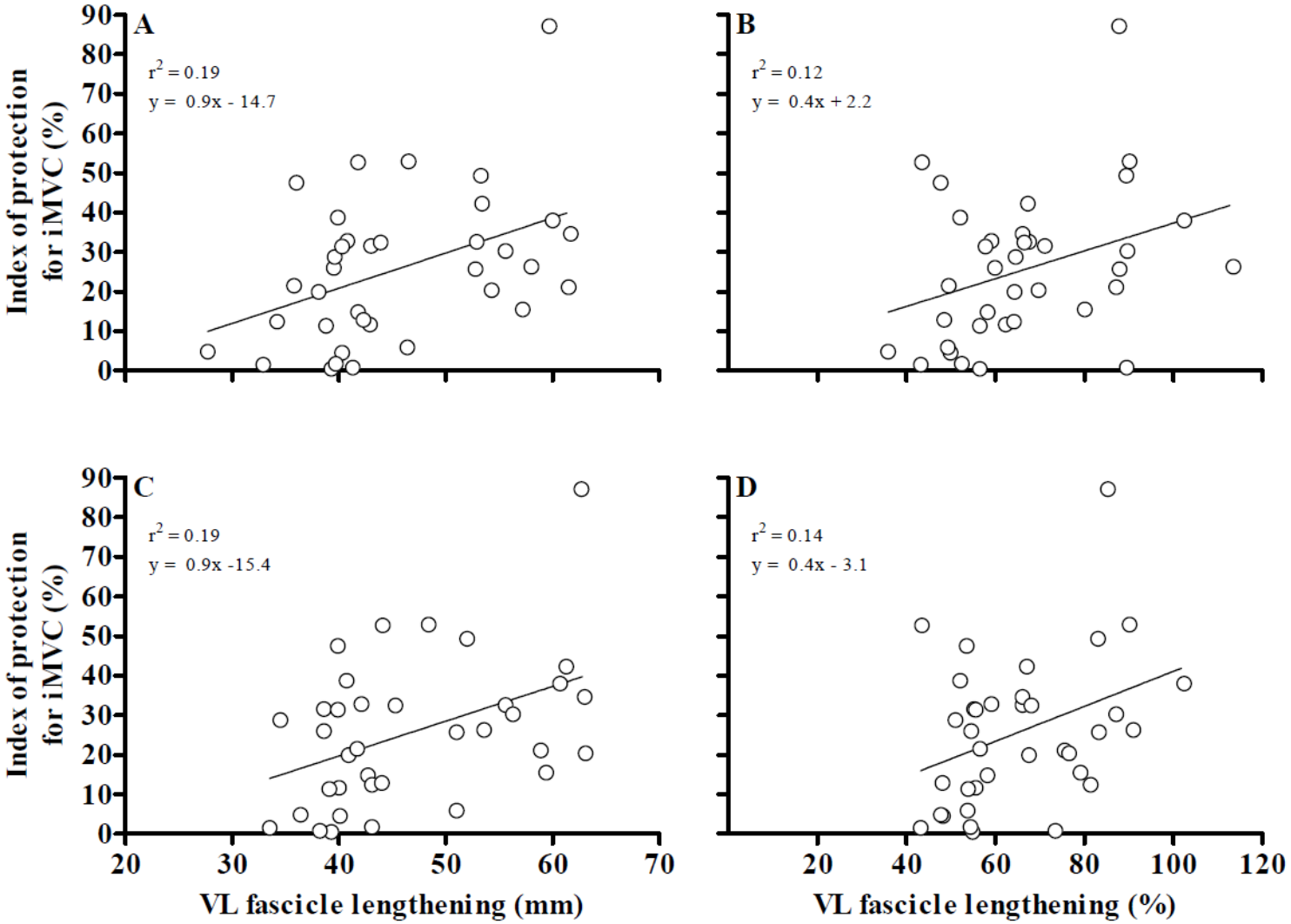

Figure 5-9 Correlation between the index of protection for maximal isometric voluntary contraction (iMVC) and absolute v*astus lateralis* (VL) fascicle lengthening (A) and relative VL fascicle lengthening (B) during the eccentric repetition performed in set 1 of the first bout of eccentric exercise. Correlation between the index of protection for maximal iMVC and absolute VL fascicle lengthening (C) and relative VL fascicle lengthening (D) during the eccentric repetition performed in set 20 of the first bout of eccentric exercise. n = 36.

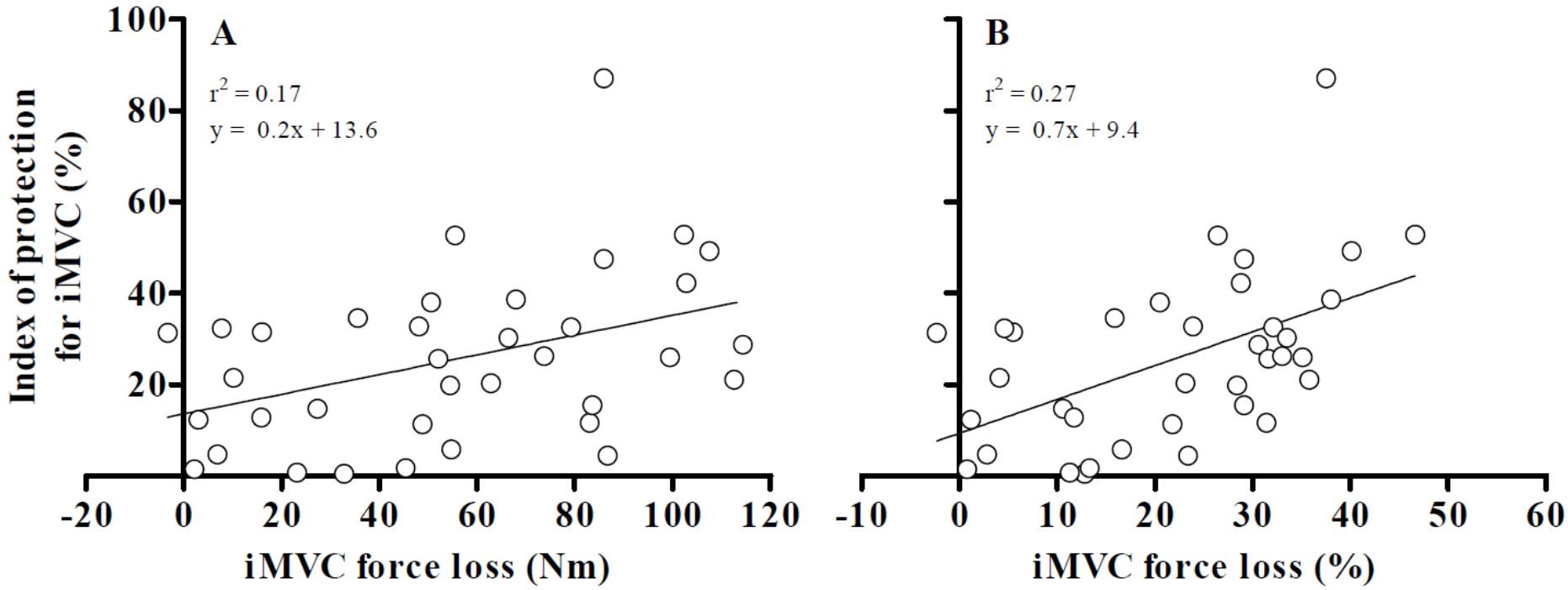

Figure 5-10 Correlation between the index of protection for isometric voluntary contraction (iMVC) and absolute (A) and relative (B) iMVC force loss at 24 h, following the first bout of eccentric exercise. n = 36.

5-5 DISCUSSION

The primary aim of this study was to assess whether adaptations in MTU behaviour during maximal lengthening contractions could explain the RBE, and whether these adaptations differed between males, eumenorrheic females, and females taking the OCP. The main findings of this study were that 100 maximal ECC repetitions of the knee extensors elicited significant responses in markers of EIMD (loss of muscle function, increased DOMS, increased blood CK and increased swelling), which were all attenuated following a repeated bout of ECC exercise performed ~ four weeks later, with no differences in the magnitude of the RBE between males and females. The RBE was accompanied by a reduced VL fascicle lengthening, which was evident at longer knee angles, despite a higher torque in the second bout, and this adaptation was consistent between males and females. A secondary aim of this study was to investigate the potential relationships between MTU behaviour and the magnitude of EIMD and the RBE. This analysis revealed associations between force loss and fascicle lengthening, such that higher fascicle lengthening in bout 1 was accompanied by a greater magnitude of the RBE in bout 2. Collectively the results of the study show that adaptations in MTU behaviour – specifically a reduction in fascicle lengthening at larger knee angles – might partly underpin the RBE, and that this adaptation is similar between, males, eumenorrheic females, and females taking the OCP.

Vastus lateralis muscle-tendon unit behaviour and the repeated bout effect

A single bout of maximal ECC exercise can trigger a profound RBE, which results in a reduction in the magnitude of EIMD markers after the second bout, compared to the first (Nosaka and Clarkson, 1995). The current study reported data consistent with the RBE phenomena; a reduced loss and faster recovery of maximal strength, a lower efflux of CK into

the blood, less soreness and swelling, and a shift in OA to a longer muscle length in B2, compared to B1. Though several mechanisms might be responsible for driving the RBE, mechanical adaptations in the MTU (Hyldahl et al., 2017) are particularly pertinent to the current study as there was both a reduction in markers in EIMD, and ~7.6% less fascicle lengthening in B2, compared to B1, both at the start (set 1) and end (set 20) of the eccentric protocol. The reduction in fascicle lengthening in B2 was present despite total ECC torque being similar, or higher, in B2 compared to B1, indicating that the changes in VL fascicle behaviour between the two bouts were independent of ECC torque. Additionally, the change in VL fascicle behaviour was not different between males and females. It is plausible to posit that the changes observed in MTU behaviour during contraction could partly explain the RBE. A reduced amount of fascicle lengthening would lead to fewer sarcomeres being actively lengthened onto the descending limb of the LT curve; this would reduce the amount of strain experienced by the sarcomeres, with sarcomere strain being denoted as a main determinant of EIMD (Lieber and Friden 1993, Talbot and Morgan 1998). This is the first study to report a reduction in muscle fascicle lengthening during a repeated bout of maximal ECC knee extension exercise coupled with an attenuation of various markers of EIMD following the second bout, in comparison to the first. This adds weight to previous research that suggest changes in MTU behaviour as a mechanism of the RBE (Lau et al., 2015, Hyldahl et al., 2017).

The reduction in fascicle lengthening on repeat bouts supports previous observations in eccentric cycling and extends these to maximal eccentric actions. Penailillo et al. (2015) reported a 16% reduction in fascicle lengthening during a repeated bout of ECC cycling, (Penailillo et al., 2015). In line with the current study, Penailillo et al. (2015) reported less muscle soreness after the second bout of exercise, with the authors indicating that the changes in fascicle behaviour were a contributor to the RBE, however, there was no difference in

isometric torque loss between B1 and B2, with both bouts failing to record a drop in torque below pre-exercise levels, making it difficult to ascertain whether a potent RBE was in fact, present. This could possibly be explained by the submaximal ECC cycling protocol used by Penailillo et al. (2015) failing to elicit sufficient damage, which has been seen in earlier submaximal EIMD research (Nosaka and Newton, 2002). Despite the lack of force deficit found by Penailillo et al. (2015), VL fascicle behaviour was altered, indicating that fascicle changes might not be dependent on the intensity of the exercise protocol used. This theory is strengthened with findings from the current study that found that VL changes appear to be independent of ECC torque, though this needs to be directly investigated.

The amount of fascicle lengthening experienced by a muscle and the subsequent EIMD response has been the focus of much research in previous years (Guilhem et al., 2011, Hoffman et al., 2014) with evidence to suggest that greater amounts of fascicle lengthening result in an exaggerated EIMD response (Guilhem et al., 2016, Hicks et al., 2017). This observation was also confirmed in the current study as a greater magnitude of fascicle lengthening was correlated with a greater amount of iMVC force loss at 24 h post-exercise after B1. In partial support, Lau et al., (2015) found a decrease in displacement of the MTJ, meaning less muscle lengthening, during a repeated bout of maximal ECC exercise of the elbow flexors, though muscle fascicle lengthening was not directly measured. Similar to the current study, Lau et al., (2015) found that markers of EIMD, including iMVC torque, recovered more quickly in B2, compared with B1, showing an RBE. Lau et al., (2015) speculated that smaller changes in MTJ displacement during the second bout of ECC exercise could have imposed less strain on the muscle fibres, thus inducing less damage. Furthermore, Hyldahl et al., (2017) theorised that the smaller changes in MTJ displacement seen in the study by Lau et al., (2015) might be due to

an increase in tendon compliance, however this has not been directly measured and therefore remains speculation.

As muscles and tendons do not act in isolation, it is important to acknowledge the contribution of various elements of the MTU when assessing muscle fascicle behaviour. As previously mentioned, Lau et al., (2015) stated that changes in tendon compliance might be responsible for the RBE, so it would be prudent to measure tendon properties directly in RBE studies investigating muscle lengthening. In the current study, tendon compliance was directly measured prior to both bouts of exercise, and there was a significant decrease in YM from B1 to B2 when standardised to the weakest participant (Figure 5-5), and the difference approached significance when YM was standardised to the lowest force produced by each individual participant (P = 0.058). As a more compliant tendon can reduce the amount of fascicle lengthening experienced during ECC exercise (Guilhem et al., 2016, Hicks et al., 2013), the reduction in YM before the second bout of exercise in the current study would seem a reasonable explanation for the reduced VL fascicle lengthening recorded in B2. However, differences in fascicle lengthening between the two bouts did not occur until a knee angle of 70°, with peak ECC torque occurring at a knee angle of 80° in both bouts. Though a difference in YM was almost evident when PT force was standardised to the lowest 'within participant' force (P = 0.058), YM was only statistically different between bouts at the lower 'between participant' force, suggesting that any differences in tendon behaviour during the two bouts of ECC exercise would occur at the start of the repetition, when forces were low and before the tendon had fully extended, at least for the participants capable of producing PT forces well in excess of the 1612 N used to standardise between participants. Although there was evidence of a trend showing that within participant PT YM was different between bouts, this was not

statistically significant. Therefore, differences in tendon compliance cannot fully explain why the reductions in VL fascicle lengthening between bouts.

Though tendons have been shown to act as a mechanical 'buffer' and protect muscle fascicles from excessive lengthening (Guilhem et al., 2016, Hicks et al., 2013), other structures might affect fascicle lengthening by increasing the passive stiffness of the muscle itself, such as the ECM. Alterations in the extra-cellular matrix between two bouts of damaging ECC exercise has previously been presented as a mechanism underpinning the RBE (Hyldahl et al., 2017), and might play a key part in both the reduced fascicle lengthening observed in B2 vs B1 of the current study and the subsequent protection against EIMD. An example of ECM modification and EIMD response was evidenced in an animal model when Lapier et al. (1995) found that following immobilisation, increased intramuscular connective tissue was present, and this attenuated an EIMD response following electrically stimulated ECC contractions. Although not induced by an initial ECC bout, the increase in connective tissue still conferred a protective effect against EIMD. Takagi et al. (2016) found that rat gastrocnemius muscle exposed to ECC contractions showed an increase in collagen expression prior to a second bout of ECC exercise, separated by 4 weeks. In comparison to a control group, rats that had been previously exposed to ECC exercise showed a lower reduction of isometric strength, demonstrating that the RBE is present, with the authors stating the increased intramuscular collagen as an explanation (Takagi et al., 2016). In human studies, a bout of ECC exercise has resulted in increased staining of VL tenascin C (Crameri et al., 2007, Raastad et al., 2010), increased NH_2 – terminal propeptide of procollagen III in the endomysium (Raastad et al., 2010), and increased lamanin-β_1 and types of type I and type III collagen (Mackey et al., 2011), with the latter study suggesting this strengthening of the connective tissue plays a role in the RBE found in the study. Taken together, evidence from both animal and human studies suggests that

intramuscular connective tissue remodelling could explain a reduction in fascicle lengthening following repeated bouts of ECC exercise and could potentially be a mechanism underpinning the RBE.

Magnitude of the repeated bout effect

The repeated bout effect typically results in participants experiencing less exaggerated markers of EIMD after completion of the second bout in comparison to the first, which is what occurred in the current study. However, when the magnitude of the RBE is assessed by the protective effect conferred by the initial bout of exercise on EIMD markers on the second (the IOP), the current study provides different results to the literature. For example, in the current study, an IOP of 24% was found for iMVC, which is smaller than previous research that reported an iMVC IOP of ~60% in the knee extensors (Chen et al., 2019). The difference in IOP in the knee extensors might be explained by the contraction type used in each study. In the current study, maximal isokinetic ECC contractions were used, whereas Chen et al. (2019) used a free-weight machine with an isotonic load of 80% maximal iMVC, throughout a range of motion of 0°-110° (0° = full extension). However, peak iMVC force losses for B1 in both studies (both occurring at 24 h post exercise) were similar at ~ 20%, therefore the magnitude of damage is unlikely to be a factor that can account for the difference in IOP between the two studies. A main difference between the current study and the study by Chen et al. (2019) was the respective four-week and two-week time periods between exercise bouts. (Nosaka et al. (2005b) displayed that the RBE is less potent when an eight-week time period is present between exercise bouts in comparison to four weeks. Therefore, it seems possible that the lower IOP evidenced in the current study in comparison to Chen et al. (2019), was due to the diminishing potency of the RBE over time. To confirm this, further research is required

investigating differences in IOP with a two- and four-week time period between identical bouts of ECC exercise.

Mediators of the repeated bout effect

A protective effect against EIMD following a maximal bout of ECC contractions can be induced by several different exercise conditions, including low volume ECC contractions (Burt et al., 2015), isometric contractions at long muscle lengths (Chen et al., 2018), and cumulative bouts of low-intensity ECC exercise (Chen et al., 2010), all of which can produce varying levels of protection against EIMD markers following a maximal bout of exercise (Hyldahl et al., 2017). What is less well understood is what factors determine the magnitude of the RBE when the two bouts of ECC exercise are identical. Two novel findings in the current study were that the IOP for iMVC was associated with a greater loss of iMVC force at 24 h after B1, and a greater magnitude of VL fascicle lengthening during B1 of ECC exercise. Moreover, there was also a positive correlation between the amount of VL fascicle lengthening and the magnitude of iMVC force loss at 24 h post exercise in B1, but this relationship was not present in B2. From these results, it appears that a greater magnitude of VL fascicle lengthening induces higher levels of force loss at 24 h post exercise, which corroborates findings showing that EIMD is greater when fascicle strain is higher (Guilhem et al., 2016). To the best of the authors knowledge, this is the first study to report that MTU behaviour is related to the magnitude of the RBE. The evidence presented indicates that greater fascicle lengthening results in greater EIMD (measured by iMVC) and this provides a more enhanced protection against subsequent damage in future exercise bouts. However, as no correlation was found between the overall reduction in VL fascicle lengthening between bouts and the IOP, it is unlikely that a reduction in VL fascicle lengthening is the sole determinant of the IOP. To further understand the

relationship between MTU properties and the RBE magnitude, additional research needs to be performed, which will be discussed further in chapter 6.

Group differences in exercise induced muscle damage and the repeated bout effect

There has been much debate in recent years as to whether sex differences are present in the magnitude of EIMD response to damaging ECC exercise. The current study does not support a sex difference in muscle damage response, with no differences detected for a range of commonly measured indirect markers of EIMD. This corroborates recent findings in the literature that no sex differences in EIMD response are present (Lee et al., 2017, Radaelli et al., 2014), even when controlled for strength, muscle size and bodyweight (Morawetz et al., 2019). Previous research has reported sex differences in the CK response to ECC exercise (Sewright et al., 2008, Oosthuyse and Bosch, 2017), including Hicks et al. (2016), who reported an increase in the CK response in males compared to females following ECC knee extension exercise. The authors stated that the differences in CK response between males and females might be due, in part, to the membrane stabilising effect of oestrogen (Wiseman and Quinn, 1994, Minahan et al., 2015). However, no such difference between males, OCP users and eumenorrheic females were present in the current study. The sex differences in CK found in the study by Hicks et al. (2016) and not in the current study might be due to the menstrual cycle phase at which the eumenorrheic females were tested at. Oestrogen peaks ~ day 14 of the menstrual cycle (671 pmol·L^{-1}) and is slightly lower during mid-luteal phase (495 pmol·L^{-1}) (Stricker et al., 2006). As the eumenorrheic females in the study by Hicks et al. (2016) were in the menstrual cycle phase where oestrogen was higher, it is possible that the cell membrane stabilising effects of oestrogen were prominent and this is the reason why sex difference in CK was found, in contrast with the current study, where participants were tested in the mid-luteal

phase. Moreover, progesterone during the mid-luteal phase is considerably higher than at ovulation [36.0 vs 2.5 nmol·L^{-1}, respectively (Stricker et al., 2006)]. Therefore, as progesterone is an antagonist of oestrogen activity (Mester and Baulieu, 1984, Hsueh et al., 1975, Hodgen et al., 1994), the cell-stabilising effect of oestrogen might have been lost, resulting in similar responses between the male and OCP using group. Although estradiol levels were not measured in males and OCP users, it was assumed that these were low as the OCP supresses oestrogen levels (Bryant et al., 2008, Fleischman et al., 2010) and males have naturally occurring lower levels of oestrogen than eumenorrheic females (Tepperman, 1987, Kendall and Eston, 2002). It is therefore likely that oestrogen had no influence on the EIMD response to ECC exercise, although testing eumenorrheic females at ovulation, when the oestrogen to progesterone ratio is at its highest (Stricker et al., 2006) might have produced different results.

To the author's knowledge, this is the first study to comprehensively investigate the difference in the magnitude of the RBE between males, OCP users and eumenorrheic females. The only difference in IOP between groups was that males had a 29.6% higher IOP for concentric strength than females in the OCP group, which was surprising, given that no differences in EIMD responses between the two groups were present for any marker of EIMD. Typically, isokinetic concentric contractions in the lower limb are deemed reliable in recording both peak torque (Timmins et al., 2016) and the torque-angle relationship (Oranchuk et al., 2020), however this might depend on how well-practiced participants are with the contraction type. Chan et al. (2020) recently reported that isokinetic strength assessment at 60°·s^{-1} required 15 contractions to produce reliable measurements, however, no current data is available for female participants, though it is unlikely that reliability would differ between males and females, considering that chapter 4 found no differences between groups in the reliability of functional measures of strength. Nevertheless, it remains possible that in B1, the male participants had

not performed sufficient repetitions to provide reliable results and the large IOP was due to a learning effect, whether this variation exists between sexes requires further research.

Conclusion

The main aim of this thesis was to use ultrasonography to further understand the properties and behaviour of the PT and VL in males and females and how these properties contribute to the EIMD response to ECC exercise and the magnitude of the repeated bout effect. To address this aim, the current study assessed whether adaptations in MTU behaviour during maximal lengthening contractions can explain the RBE, and whether these adaptations differ between males, eumenorrheic females, and females taking the OCP. An additional aim of this study was to investigate the potential relationships between MTU behaviour and the magnitude of EIMD and the RBE. It was found that a bout of 100 maximal ECC repetitions of the knee extensors elicited significant responses in markers of EIMD which were all attenuated following a second bout of ECC exercise performed ~ four weeks later, confirming the RBE. *Vastus lateralis* fascicle lengthening during ECC exercise was reduced in the RBE, in comparison to the first. This change might, in part, be attributed to an increase in patella tendon compliance as Young's modulus was reduced prior to the second bout of ECC exercise. Furthermore, the amount of fascicle lengthening experienced during the initial bout of exercise correlated with both the magnitude of isometric force loss at 24 h post-exercise and the magnitude of protective effect against isometric force loss following a repeated bout of ECC exercise. Finally, VL MTU behaviour did not differ between males, OCP using females, and eumenorrheic females, nor did the EIMD response following an initial and repeated bout of maximal ECC exercise, with the magnitude of the protective effect remaining similar between groups. These data show that reduced muscle fascicle lengthening during a second bout of ECC knee extension exercise is a

mechanistic underpinning of the repeated bout effect, thus addressing the main aim of this thesis.

CHAPTER 6 GENERAL DISCUSSION

6-1 INTRODUCTION

The overall aim of this thesis was to examine the properties and behaviour of the PT VL at rest and during repeated bouts of maximal ECC exercise using US and investigate how this relates to the EIMD response. To achieve this, three studies were designed and constituted the experimental chapters within this thesis. Chapter 3 aimed to establish the reliability and validity of US measurements of the patella tendon by comparison to the "gold standard" MRI. It was established that US showed excellent agreement with MRI when used to assess PT CSA. Moreover, US derived measures of PT CSA showed excellent test re-test reliability, irrespective of operator experience. Chapter 4 aimed to determine the reliability of using US to quantify PT properties at rest and during maximal isometric exercise, and VL properties at rest and during maximal ECC exercise. The results from chapter 4 established that US could be used to measure VL and PT properties at rest and during maximal exercise with excellent test-re-test reliability. Moreover, reliability was consistently excellent for males, OCP using females and eumenorrheic females. Chapter 5 assessed whether adaptations in MTU behaviour during maximal lengthening contractions could explain the RBE, and whether these adaptations differed between males, eumenorrheic females, and females taking the OCP. It was found that VL muscle fascicle lengthening was lower during a repeated bout of ECC exercise and this was associated with lower reductions in muscle force at 24 h post-exercise. Moreover, the reduced muscle fascicle lengthening might be attributed to a reduction in PT Young's modulus at low patella tendon forces. Finally, there were no between group differences in MTU behaviour or EIMD response between males, OCP using female, and eumenorrheic females following ECC exercise in either bout. This chapter will discuss the main findings of this thesis in relation to the existing literature.

6-2 MAIN FINDINGS

6-2.1 Ultrasound as a measurement tool for measuring muscle-tendon unit properties at rest and during exercise.

Over the last few decades, the use of 2D B-mode ultrasonography has been used to assess the architectural and mechanical properties of human skeletal muscle and tendons. This has allowed the *in vivo* measurement of real-time MTU mechanics during various activities, which has led to a greater understanding of how MTU properties can influence human performance (Massey et al., 2017), injury risk in sport (Ribeiro-Alvares et al., 2020), and the EIMD response to strenuous exercise (Hicks et al., 2016, Hicks et al., 2017). Any measurement tool needs to be subjected to rigorous testing to ensure that valid and reliable results are obtained, and US is no different. Chapter 3 showed that US had excellent agreement with MRI when measuring PT CSA and that US produced reliable results over repeated measures, irrespective of operator experience. Furthermore, chapter 4 showed that US measures of resting and dynamic VL MTU properties were also reliable over multiple measures. Understanding the variability in the measures obtained in chapter 4 allowed the interpretation of results from chapter 5 to be made with confidence. However, the measures that were tested for reliability (chapters 3 and 4) and to assess the MTU behaviour over repeated bouts of exercise (chapter 5), were chosen due to the limitations relating to available equipment and their respective capabilities. Though the chosen measures had been used in previous literature, supplementary methodologies might be able to address some of the areas that this thesis was unable to answer.

The constituent structures of the muscle-tendon unit can be measured independently using US (Fukunaga et al., 1997), but the simultaneous measurement of the muscle and tendon is more difficult. Previous research has shown that fascicles are actively lengthened to a lesser extent during ECC contractions when tendon compliance is higher (Guilhem et al., 2016, Hicks et al.,

2013), however, the direct measurement of the tendinous contribution to these ECC contractions has not been directly assessed. Hicks et al. (2013, 2017) directly measured PT elongation during a ramped iMVC and calculated tendon stiffness and YM based on these measures. However, direct assessment of the PT was not conducted during ECC exercise and the relationships between PT compliance and VL fascicle lengthening reported by Hicks et al. (2013) were based on the ramped iMVC data and a *post hoc* calculation of VL excursion, that estimated total MTU lengthening (Spoor et al., 1990). Similarly, Guilhem et al. (2016) based the tendinous contributions to total MTU behaviour during ECC plantar flexor exercise on indirect calculations, displaying that a compliant tendon meant that fascicles lengthened less. In chapter 5, no relationships were found between tendon properties and VL fascicle lengthening, based on VL PT properties during a ramped iMVC. However, across two bouts of identical ECC exercise, VL fascicle behaviour changed significantly in the absence of PT compliance changes at high forces. Though alternative explanations were presented to explain the changes in VL behaviour between exercise bouts, there is a possibility that the tendon behaviour did change, but the methodology implemented did not allow these changes to be detected. Although speculative, had PT elongation been measured during the ECC exercise, differences in PT lengthening, stiffness and/or YM might have been detected. However, to date, no studies have directly measured PT behaviour during maximal ECC contractions, meaning that any muscle-tendon interactions can only be indirectly inferred.

Whilst it would be logistically difficult, directly assessing muscle and tendon behaviour simultaneously is possible. For example, using two ultrasound machines synchronised with the same torque acquisition system, Massey et al. (2017) implemented the concurrent recording of VL fascicle and PT behaviour during ramped contractions, allowing the calculation of absolute MTU stiffness and the contributions of the VL and PT, independently. In theory, the same

methodology could be implemented during ECC contractions, which would allow a more accurate interpretation of the contribution of both the PT and the VL to total MTU behaviour. Should this be achieved, additional methodological considerations would need to be employed. For example, PT tendon stiffness and by extension, PT YM is calculated using PTMA (Onambele et al., 2007), which has been shown to change during knee flexion (Wretenberg et al., 1996, Tsaopoulos et al., 2006, Krevolin et al., 2004). This would then require the measurement of PTMA over the range of motion to be used in the exercise, so that appropriate tendon forces could be calculated at a given knee joint angle. As was found in chapter 5, VL fascicle lengthening between two identical bouts of ECC exercise did not differ until a knee angle of 70°, therefore having the means to distinguish the contribution of the tendon at, and beyond this point might ascertain why the differences in VL lengthening between bouts were occurring. Unfortunately, measuring PTMA over a range of motion is difficult and either requires a series of static images measured over several joint angles (Wretenberg et al., 1996, Hashizume et al., 2012), or real-time MRI (Fiorentino et al., 2013). Moreover, high force contractions, albeit isometric, have been shown to increase joint moment arm by 22-44% (Maganaris et al., 1999, Maganaris et al., 1998), which implicates the calculation of tendon stiffness further. Whilst dynamic measurement of PTMA is achievable at low forces (Fiorentino et al., 2013), it remains unclear if it is possible to measure PTMA at higher forces, given the limitations of MRI scanning logistics. Nevertheless, future research should seek to implement more joint specific measures of PTMA and PT elongation, so that a better estimation of mechanical tendon properties can be obtained at specific joint angles during dynamic movement.

Young's modulus is reliant on the measurement of tendon CSA (Onambele et al., 2007), which can be accurately and reliably obtained via US, as reported in chapters 3 and 4. At present,

calculations of YM are based on resting PT CSA at a fixed knee angle (Onambele et al., 2007) and therefore do not include potential variations of PT CSA as a result of strain. In chapter 5, at low PT forces, YM was reduced prior to the second bout of ECC exercise, in comparison to the first bout and it was stated that this difference might contribute to the reduction in VL fascicle lengthening seen during the second bout of ECC exercise, in comparison to the first. However, as previously discussed, this interpretation is based on two different contraction modalities and at a fixed knee angle. To more accurately understand the contribution of changes in YM to changes in fascicle lengthening, more appropriate measurement of PT CSA could be used to calculate YM. Due to the crimped region on the tendon (Whittaker and Canham, 1991, Stouffer et al., 1985), the diameter of the PT would not conform to Poisson's ratio (Poisson, 1827, Greaves et al., 2011), whereby the diameter would decrease at a constant to the strain. As US is a reliable tool for measuring CSA, it seems feasible that measuring PT CSA under strain is another area of research that could be conducted. To date, this is an area of research that has yet to be explored but would contribute to the more accurate interpretation of the relationship between tendon compliance and fascicle behaviour.

In summary, the use of US technology to measure muscle and tendon properties has allowed the studies in chapter 4 and 5 of this thesis, and several other studies, to explore the intricate relationship within the MTU, with chapter 5 exploring how this relationship changes upon exposure to maximal ECC exercise. However, alterations in methodological approach, such as measuring tendon behaviour during ECC exercise, might allow researchers to further understand these MTU interactions. Through this approach, the findings form this thesis would be strengthened, and it would create a platform for future research opportunity.

6-2.2 The role of the muscle tendon unit and the magnitude of EIMD response

As stated in section 2-6, several key determinants are purported to be responsible for eliciting EIMD, and the results from chapter 5 add some interesting data to this debate. One proposed determinant of EIMD is the length at which the muscle is exercised. Specifically, ECC exercise at longer muscle lengths elicits a greater magnitude of EIMD (Nosaka et al., 2005a, Newham, 1988). Chapter 5 showed that a greater amount of VL fascicle lengthening correlated with a greater amount of iMVC force lost at 24 h post-exercise after the first ECC bout, thereby supporting the claim that ECC exercise at longer muscle lengths elicits more damage. However, as all of the participants in chapter 5 exercised through the same range of motion, it would appear that the measurement of muscle length needs to go beyond the amount of degrees of rotation that the limb has travelled through. The findings from chapter 5 support previous research that has reported that greater fascicle strain over the same range of motion is correlated with a greater amount of iMVC force loss (Guilhem et al., 2016). This is contrary to the findings of Hicks et al. (2016), who found no relationship between the degree of VL fascicle lengthening and iMVC force loss, however a relationship was found for CK efflux, which was not present in chapter 5. Using CK to determine structural damage following ECC exercise is difficult, as individual responses can be highly variable and do not correlate well with other markers of EIMD (Nosaka and Clarkson, 1996, Heled et al., 2007, Fridén and Lieber, 2001). However, a loss in maximal force has been suggested as a highly valid and reliable marker of EIMD in humans (Damas et al., 2016, Warren et al., 1999) and might be a better indicator of damage to the muscle, in the absence of direct techniques. In chapter 5, an end range of motion of 110° knee angle was used, in comparison to 90° used by Hicks et al. (2017); the participants in chapter 5 would most likely have had more sarcomeres actively lengthened further down the descending limb of the LT curve, exposing a greater number of weaker sarcomeres to stress

and damage, as explained by the popping sarcomere theory (Morgan, 1990, Morgan and Proske, 2004). It would therefore appear that ECC exercise to a knee angle of at least 110° is required to cause iMVC force losses that are attributable to the amount of fascicle lengthening in the VL.

As the muscle and tendon are both constituents of the wider MTU, the behaviour of both tissues must be considered when evaluating the involvement of MTU behaviour in EIMD. Chapter 5 uncovered some results that are inconsistent with the literature. For instance, despite the relationship between greater VL fascicle lengthening and greater iMVC loss, there was no such relationship for any measured tendon properties. It has been reported that the tendon can act as a mechanical buffer, thereby protecting the muscle from excessive lengthening and subsequent damage (Guilhem et al., 2016, Reeves and Narici, 2003). However, the results from chapter 5 showed that no relationship existed between PT properties (stiffness and YM) and either the magnitude of VL fascicle lengthening or the magnitude of EIMD. Previous research that has shown that tendon properties influence fascicle lengthening in the VL (Hicks et al., 2013, Penailillo et al., 2015) or plantar flexors (Guilhem et al., 2016) but this work is not entirely reflective of the possible role of the tendon in ECC muscle actions. For example, Guilhem et al. (2016) and Penailillo et al. (2015) estimated tendon contribution to total MTU lengthening. As in chapter 5, Hicks et al. (2013) used tendon properties derived from ramped iMVCs to investigate potential relationships between PT properties and VL fascicle lengthening during ECC exercise. Though differences between chapter 5 and the study by Hicks et al. (2013) were present for the influence of tendon properties on VL fascicle lengthening, chapter 5 and another study by Hicks et al. (2016) are in agreement that PT properties do not correlate with markers of EIMD. Within the literature, there appears to be a disconnect between tendon properties, fascicle lengthening and the magnitude of EIMD following ECC exercise. As discussed earlier

in this section, chapter 4 showed that ramped iMVCs using US to measure tendon elongation is a reliable tool for estimating PT properties. However, PT properties derived from this method might not translate fully to the properties and behaviour of tendons during ECC actions. Most notably, the differences in contraction mechanics, such as the role of titin increasing muscle stiffness during ECC muscle actions and not in isometric contractions (Herzog, 2018), might mean that tendon behaviour during isometric contractions differs from that during ECC contractions. This could explain why differences in VL fascicle lengthening between bouts of ECC exercise in chapter 5 were present, in the absence of differences in PT properties at high forces. As discussed in section 6-2.1, there is potential to measure PT properties during ECC exercise, and this could be used to further understand the role of the tendon as a determinant of EIMD and its action as a mechanical buffer.

6-2.3 Muscle-tendon adaptations as a mechanism of the repeated bout effect

The repeated bout effect, characterised by a reduction of muscle damage following a second bout of ECC exercise in comparison to the first (Nosaka and Clarkson, 1995), is thought to be due to a multitude of mechanisms, one of which being alterations in MTU behaviour between bouts (Hyldahl et al., 2017). Chapter 5 provided evidence that VL fascicle lengthening was reduced during ECC exercise during the second bout, in comparison to the first. However, some previous research has found no differences in fascicle behaviour between two bouts of backwards downhill walking, despite an RBE being evidenced through the attenuation of EIMD markers following the second bout (Hoffman et al., 2016). Nevertheless, the reductions in VL fascicle lengthening during bout 2 in chapter 5 was accompanied by a reduction in the magnitude of EIMD in bout 2 in comparison to bout 1, indicating that the RBE was present. The reduction in muscle lengthening during the second bout of ECC exercise was consistent with previous research, though a mechanism behind this reduction in muscle strain was not

determined (Lau et al., 2015, Penailillo et al. 2015). Hyldahl et al., (2017) did speculate that alterations in tendon properties between bouts could be an explanatory factor for the reductions in muscle lengthening seen in the second bout, although this remains a theory. Though chapter 5 did report a difference in YM prior to bout 2 in comparison to bout 1, this was only for calculations standardised to the lowest tendon force across all participants, and no difference in YM was reported when the standardisation was to the lowest tendon force per participant. Therefore, an alternative explanation for the reduction in VL fascicle lengthening in bout 2, must be involved.

To determine what was driving the reduction in VL fascicle lengthening and markers of EIMD reported in bout 2 of chapter 5, adaptations in other aspects of the muscle structure following ECC exercise must be explored. For example, titin can increase muscle stiffness by binding to actin in the presence of Ca^{2+} during ECC contractions (Herzog, 2018). Moreover, titin-based elastic forces have been shown to maintain A-band centering within the sarcomere during ECC contractions, maintaining force generating capacity via the maintenance of a more optimal actin-myosin overlap (Horowits and Podolsky, 1987). Following damaging ECC exercise, immunogold staining demonstrated a fragmentation of titin, evidenced by a dislocation of the COOH terminus of titin towards the A-band and H zone of the sarcomere (Macaluso et al., 2014). Additionally, urinary fragments of titin have been detected in response to EIMD (Yamaguchi et al., 2019, Kanda et al., 2017), suggesting that titin becomes damaged following ECC exercise. An interesting finding by Yamaguchi et al. (2019) was that titin fragments did not appear in the urine following a repeated bout of ECC exercise. Due to the time-course similarities to CK release into the bloodstream following the first bout of ECC exercise, and the lack of titin fragment increase following the second bout, Yamaguchi et al. (2019) suggested that the reason why urinary titin fragments were not increased following the second

bout was possibly due to reductions in sarcolemma damage, thus preventing intracellular titin fragments from escaping, similar to how levels of CK are reduced following repeated bouts of ECC exercise (Lehti et al., 2007). Though this is possible, an alternative explanation for the reduction in urinary titin fragments following the bout could be due to changes in titin properties following the first bout of ECC exercise, though this has yet to be investigated in humans.

Research investigating the role of titin stiffness in ECC muscle actions and the response of titin to EIMD has become popular over the last few years. For example, a significant increase in the relative phosphorylation of the elastic PEVK-domain of titin was demonstrated in rats subjected to a single ECC exercise bout, which is expected to result a higher titin-based stiffness within the muscle (Müller et al., 2014). Additionally, increased titin phosphorylation, and an indication of increased titin stiffness, was also observed following three weeks of running in mice (Hidalgo et al., 2014). Although an increase in titin stiffness following exercise is yet to be confirmed in humans, the findings from Müller et al. (2014) and Hidalgo et al. (2014) might explain observations in chapter 5 and in a study by Penailillo et al. (2015). For example, both chapter 5 and Penailillo et al. (2015) observed a reduction in VL fascicle lengthening upon a second bout of identical ECC exercise. In chapter 5, the lack of differences in tendon compliance at high forces between bouts suggests that changes in PT properties cannot fully explain the VL changes, although it must be noted that tendon compliance was measured during a separate iMVC and not during the ECC exercise. An alternative theory is that an increase in titin stiffness between bouts is responsible for the reduction in VL fascicle lengthening observed during B2 in chapter 5. At the start of the contraction, the compliant Ig domain of titin extends first followed by the PEVK region (Linke et al., 1998), which is the region where increased titin stiffness is suggested to occur (Müller et al., 2014, Hidalgo et al.,

2014). Therefore, if changes in titin stiffness would not have an effect until the PEVK region is stretched, it would seem feasible that muscle lengthening would remain similar at shorter muscle lengths where only the Ig regions of titin were being lengthened. This would explain observations in chapter 5, where a reduction in VL fascicle lengthening during B2, compared to B1, was only observed at knee angles of over 70°. Though an interesting theory, changes in titin stiffness and the effect on muscle lengthening between two bouts of ECC exercise have yet to be studies in humans and therefore, this theory remains speculative.

A dominating theory of why the RBE occurs would be developed if a specific mechanism was consistently observed across all RBE studies, however, as this is not the case, it appears as though the RBE is very much a multifactorial phenomenon. For example, in chapter 5, although the amount of VL fascicle lengthening was correlated with the magnitude of the protective effect for iMVC, the difference in total fascicle lengthening between the two bouts was not. Essentially, this implies that MTU behaviour, or fascicle lengthening specifically, is more aligned with the magnitude of iMVC force loss following the initial bout of ECC exercise and it is the magnitude of EIMD that dictates the IOP following the second bout of ECC exercise, as there was no relationship between IOP and the difference in total fascicle lengthening between bouts. It therefore seems apparent that magnitude of the RBE is dictated by the magnitude of initial EIMD following the initial bout of exercise, a notion that has been supported by previous literature (Nosaka, 2011). However, until the direct measurement of both tendon and fascicle behaviour is measured, the definitive role of the MTU in the RBE cannot be ascertained.

Although this thesis presents evidence for the role of the MTU in the RBE, changes in muscle and tendon during ECC exercise cannot fully explain the RBE and several questions remain.

For example, the contralateral RBE, whereby damage to one limb confers a protective effect to the limb on the opposite side of the body (Tsuchiya et al., 2018, Howatson and van Someren, 2007), is unlikely to be due to local muscle adaptations, such as sarcomerogenesis (Proske and Morgan, 2001). Additionally, a protective effect against maximal ECC exercise can be conferred by isometric contractions that produce very little EIMD (Chen et al., 2012a, Tseng et al., 2016), which means adaptations due to structural remodelling are unlikely. Consistent with the conclusion by Hyldahl et al. (2017), the RBE remains a multi-faceted phenomenon, comprised of neural, inflammatory, extracellular matrix and MTU adaptations. However, the contribution of each mechanism remains unknown.

6-3 PRACTICAL IMPLICATIONS AND FUTURE RESEARCH

The results of this thesis could have a number of important practical and research implications. For example, the longer time period between one exercise bout and the next reduces the amount of protection conferred by the RBE (Nosaka et al., 2005b). Therefore, should athletes take an extended off -season, they would be more susceptible to EIMD and potentially more severe injuries (Emery and Meeuwisse, 2001, Brumitt et al., 2020). The findings from chapter 5 illustrate that ECC exercise that requires exercise at a longer muscle length produces more fascicle strain and more EIMD. Though more EIMD results in a greater amount of protection against damage in a repeat bout, results from chapter 5 show that muscle function had still not recovered after seven days. Though the protective effect is greater with more muscle lengthening and EIMD, waiting seven-days to perform another exercise session would be impractical in most sporting scenarios. Therefore, a number of options are available to strength and conditioning coaches to minimise EIMD and recovery time needed. One option would be to choose exercises that limit muscle length, quarter, or half squats as an example. This would reduce excessive fascicle lengthening that chapter 5 and other research has showed to impair

muscle function (Guilhem et al., 2016). Using findings from RBE literature beyond this thesis could also be implemented. For example, coaches could use a number of sub-maximal sessions to confer a protective effect against maximal exercise (Chen et al., 2010), though the protective effect is likely to diminish over time (Chen et al., 2012a), or implement maximal isometric exercises at long muscle lengths to give athletes some protection against EIMD from ECC exercise without the associated muscle soreness and decrements in function (Tseng at al., 2016, Chen et al., 2012b). Although individual responses would vary, implementing some of these strategies could allow more frequent training to occur without functional impairment.

In terms of future research directions, this thesis provides a number of avenues that could be explored. The current thesis has focussed heavily on the use of the in *vivo* measurement of VL and PT properties using US. An immediate question that needs answered is how the tendon behaves during single and repeated bouts of ECC exercise. As discussed throughout this final section, current contributions of the tendon to total MTU lengthening remains either via a prediction (Guilhem et al., 2016, Penailillo et al., 2015) or via extrapolation from isometric contractions (Hicks et al., 2013). The simultaneous measurement of PT and VL fascicle behaviour, like that implemented by Massey et al. (2017), should be considered and this would give a much better understanding of the muscle-tendon interaction during ECC exercise.

In section 6-2.3, the theory that titin stiffness changes between bouts was proposed as a potential mechanism behind the reduced VL fascicle lengthening seen in bout 2. Recently, the action of titin and the role that it plays in EIMD has become an area of speculation. The binding of titin to actin during active lengthening is proposed to alleviate the strain on the contractile filaments (Herzog, 2018, Herzog, 2014). The potential remodelling of titin following EIMD and the increased stiffness in titin's PEVK region (Hidalgo et al. 2014, Müller et al. 2014)

provides a hypothesis that might explain the reductions in fascicle lengthening seen in a repeat bout of ECC exercise. Although this would be a difficult hypothesis to test, combining this with the concurrent measurement of VL and PT during ECC would provide a more holistic understanding of the interactions between the active and passive elements of the MTU.

Finally, a key theme to this thesis was the investigation of sex differences and the role of the OCP in EIMD and the RBE. Results from chapter 4 showed that US measures of MTU behaviour during ECC exercise were reliable for males and females. In chapter 5, it was shown that no differences in MTU measures existed between males, eumenorrheic females and females using the OCP, which contradicted findings from Hicks et al. (2013, 2016) and that this might be due to the point in the menstrual cycle that the eumenorrheic females were tested, as discussed in section 5-5.4. To date, only one study has investigated EIMD across various phases of the menstrual cycle and found that the inflammatory response was greater in the mid-follicular phase in comparison to the mid-luteal phase (McKinley-Barnard et al., 2018). The authors attributed this to oestrogen exerting a cyto-protective effect on the sarcolemma. However, the results of this study might have been confounded by the contralateral RBE (Howatson and Van Someren 2007), as the mid-follicular session was always performed first, with the mid-luteal session performed on the contralateral limb. Both the RBE and contralateral RBE make testing the same eumenorrheic female participants on multiple occasions problematic, as there is no guarantee a protective effect will have been induced unless a six month period in-between visits is employed, which is the longest known time an RBE has been proven to exist (Nosaka et al., 2001). Therefore, to fully understand the effect of menstrual cycle phase on EIMD susceptibility, a study testing groups of eumenorrheic females at specific menstrual cycle phases could be employed, with enough females per phase to ensure statistical power. Given the high intra-individual variability of EIMD response, this would require a large

sample size. This could also be extended to investigate the effect of menstrual cycle phase on the magnitude of RBE. Once established, this would give researchers a more solid basis to investigate sex differences in EIMD, which still remains unclear in the literature whether they exist.

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APPENDICES

APPENDIX 1 – EXAMPLE OF A PARTICIPANT INFORMATION SHEET AND INFORMED CONSENT

Study Title: The influence of sex and the oral contraceptive pill on markers of exercise induced muscle damage and the repeated bout effect, following maximum, single limb, knee extension exercise

Investigator: Mr. Steven Marshall

Participant Information Sheet

You are being invited to take part in this research study. Before you decide it is important for you to read this leaflet so you understand why the study is being carried out and what it will involve.

Reading this leaflet, discussing it with others or asking any questions you might have will help you decide whether or not you would like to take part.

What is the Purpose of the Study?

Following a bout of unfamiliar exercise, human muscle can become damaged. Following this, adaptations occur so that when the same exercise is performed again sometime later, the muscle becomes less damaged when compared to the initial bout. This is known as the repeated bout effect and the cause is not fully understood. The focus of our research is to see what changes happen in the muscle and tendon (tissue that connects muscle to bone) before, during and after the unfamiliar bout of exercise, and then again when it is repeated. To measure these changes, we will use ultrasound imaging and an isokinetic dynamometer. There has also been some research to suggest that the response to this type of exercise might differ between males and females. Furthermore, females that take the oral contraceptive pill might respond differently to females who don't use any type of hormone influencing contraception
The results of this study will provide evidence of how the muscle and tendon responds to this repeated exposure to unfamiliar exercise. In addition, we will be able to make comparisons over muscle and tendon properties between males and females, which is a largely unexplored component of sport science research.

Why have I been invited?

You have been invited to take part in this study as you are aged between 18-35 with no current musculoskeletal injuries and are currently exercising at least twice per week. Female participants will have reported no abnormalities in their menstrual cycle, as assessed via a confidential menstrual cycle questionnaire. Abnormalities include such things large fluctuations between the length of your cycle, cycles that last upwards of 45 days and menstruation that lasts longer than 7 days. You therefore meet the inclusion criteria for the study and are invited to participate.

Do I have to take part?

No. It is up to you whether you would like to take part in the study. I am giving you this information sheet to help you make that decision. If you do decide to take part,

remember that you can stop being involved in the study whenever you choose, without telling me why. You are completely free to decide whether or not to take part, or to take part and then leave the study before completion.

What will happen if I take part?

Prior to testing, you will be asked to complete pre-screening health and menstrual cycle (females only) questionnaires which will ask you to disclose information about your health. You will be asked to attend the laboratory on thirteen separate occasions, the first being a practice session (visit 1). Then you will have your first experimental visit (visit 2), followed by follow up sessions (visits 3-7) at 24, 48, 72, 96 and 168 hours later. You would then repeat this about 4 weeks after your first experimental visit (visits 8-13). Visits will take place in the neurophysiology lab on the 4th floor of the Northumberland Building, room NBD415. You will be asked to refrain from strenuous exercise and consuming alcohol in the 24 hours prior to testing and throughout the follow up visits. On the day of testing, you will be asked to refrain from consuming caffeine, non-steroidal anti-inflammatory drugs (e.g. ibuprofen) and other stimulants. In between visits, you will be asked to maintain your regular eating habits and keep a daily exercise diary. Details of the visits are given below.

Visit 1 – Familiarisation session

During the familiarisation session, you will be habituated with the study protocol and the techniques used to measure how your muscle behaves during contraction. Before this, the researcher will take your height, weight and thigh circumference, and a scan of your knee using a very low dose X-ray. The procedure familiarisation will involve you sitting in a device called a dynamometer. Then, with your knee set at a 70° angle (0° being fully extended), you will perform a short warm up, then push as hard as you can against the lever arm of the device for a few seconds, this is known as an isometric maximum voluntary contraction. You will repeat this at an 80° knee angle, to experience the differences in pushing with your leg slightly more bent. Finally, you will be demonstrated 5 eccentric contractions (the muscle is producing force as it gets longer). The researcher will start with their leg almost straight, followed by the lever of the device slowly forcing the leg to bend whilst they try to resist as much as possible. This type of movement is called a maximum eccentric contraction. The Familiarisation session should last between 20-30 minutes.

Visit 2 – Assessment of muscle and tendon behaviour during maximal contractions of the quadriceps and bout 1 of 'muscle damaging' exercise

The session will begin by the researcher locating the centre of the quadricep muscle on the outside of your leg, using an ultrasound probe. Ultrasound images of your patella tendon will be taken by placing a probe on the knee. A conductive gel will be used to enhance the image. Images will be taken at rest, during passive movement and during maximal isometric contractions. You will then be asked complete assessments of lower limb muscle soreness and pain pressure threshold (how much soreness you feel when a specific amount of pressure is applied). A capillary blood sample taken from the ear lobe will then be used to measure a specific compound (creatine kinase) assessed during muscle damage studies. If your creatine kinase score is outside of the detectable range, you will be asked to give a venous blood sample, taken from the arm. Ultrasound images of the quadriceps muscles will then

be taken during maximal and sub-maximal isometric contractions at 90°, 85°, 80°, 75°, 70°, 65° and 60° knee angles. You will then be asked to perform maximal and sub-maximal contractions of the hamstrings at a 90° knee angle. You will then perform 3 concentric contractions of the quadriceps. For the 'muscle damaging' exercise, you will perform 15 sets of 10 maximal eccentric contractions of the quadriceps, against the lever arm of the dynamometer. Ultrasound images of the quadriceps will be taken during these contractions. Following this, you will perform follow up measures including 4 isometric contractions and 2 concentric contractions of the quadriceps, thigh circumference, muscle thickness, a capillary blood sample and measures of muscle soreness. The first experimental visit is expected to take between 2-2.5 hours. For female participants using oestrogen stabilising forms of contraception (e.g. the oral contraceptive pill), the muscle damage visit will occur on day 21 of the menstrual cycle (day zero will be reported to the researcher). Females who are not using any form of hormone-based contraception will be provided with home ovulation kits to be used from day 9 of their menstrual cycle. This is done by either urinating on the test strip of the ovulation kit for 5-6 seconds or dipping the test strip into a collected sample of urine for 15 seconds. This test can be done at any time of day, but the time must be consistent between days and the sample should be collected following a period of 4 hours without urination. When peak levels of luteinising hormone are confirmed by the test kits on 2 consecutive days (by the appearance of a 'smiley face' on the kit), the first muscle damaging testing visit will occur 7 (± 2) days after this.

Visits 3, 4, 5, 6 and 7 – 24, 48, 72 96 and 168 hour post-damage follow up sessions
All of these sessions will follow the same format and will consist of a capillary blood sample, measures of muscle soreness and pain pressure threshold, muscle circumference and thickness, 4 isometric contractions and 3 concentric contractions of the quadriceps. Each follow up session is expected to last 20-30 minutes and will be performed at approximately the same type of day

Visits 8, 9, 10, 11, 12 and 13 – Second muscle damaging visit and follow up sessions
Approximately 4 weeks after the first 'muscle damaging' visit, you will return to the lab and repeat visit 2 in an identical fashion. In the days post, you will repeat the exact same follow up sessions you did after the first 'muscle damaging' visit. For female participants, the same procedures (described earlier) will be in place to ensure that testing is performed at the correct stage of the menstrual cycle.

What are the possible disadvantages of taking part?
Due to the nature of the eccentric exercise bout, you will likely experience severe soreness in the exercised leg. There is also some injury risk associated with the contractions involved in the study, however a warm up will be performed in order to minimize risk. If injury does occur, appropriate sports medicine treatment will be organised. The X-ray scan, known as a DEXA, will expose you to a very low dose of radiation, however this is less than 2 days' exposure to natural background radiation. For comparison, a flight from the UK to North America is the equivalent to a week's exposure to natural background radiation. The ultrasound procedure is non-invasive and should not cause any discomfort. Hypoallergenic conductive gel is used to enhance the signal. This gel is cold when applied but this sensation will only last a few

seconds. The probe is placed on the area of interest with minimal pressure and images are transferred to a computer.
Venous and capillary blood sampling may result in some light, local bruising, and slight physical discomfort for the participant. This will be minimized by good practice and samples being taken by a trained phlebotomist. Standard precautions will be taken, such as wearing gloves, and consumables will be disposed of as per clinical waste guidelines.

What are the possible benefits of taking part?
By participating in the study, you will be participating in scientific research that aims to demonstrate the effect of sex and oral contraceptive pill use muscle damage and the repeated bout effect. You will gain experience and knowledge of how an experimental study is operationalized and will receive results of basic strength testing.

Will I be reimbursed for participating in the study?
Due to the large time commitment and the nature of the study, you will be given a £50 Amazon voucher on completion of the study.

Will my taking part in this study be kept confidential and anonymous?
All data storage will abide by the universities Data Management Procedures. All data will remain anonymous to anyone other than the researcher and supervisor. A coded system will be used to ensure anonymity. All paperwork will be coded apart from your consent form, which will be stored in a secure filing cabinet in a lockable office to ensure confidentiality.

How will my data be stored?
All data will be stored on a secure, password protected computer system. All paper data will be stored in a lockable unit. Data will be backed up on a password protected external hard drive. All data will be stored by the lead researcher, in accordance with Universities Data Management Procedures. All data will be destroyed 5 years after data collection.

What will happen to the results of the study?
On completion of the study results will be collated with those of the other participants and will in no way be linked back to you. Findings may be published in scientific papers or presented at conferences. If you would like a copy of the summary of findings you may contact the researcher at the email address given below and you will be issued a copy once the study has ended. Please note that individual data will not be issued, and collated findings will not be available immediately after completion of the study.

Who is Organizing and Funding the Study?
The study will be organized and funded by Northumbria University and sport England TASS.

Who has reviewed this study?
This study has been reviewed and approved by the Faculty of Health and Life Sciences Research Ethics Committee at Northumbria University. Should you require any information about the ethical aspects of this study please contact;

Dr Mick Wilkinson, Northumberland Building, Northumbria University, Newcastle upon Tyne, NE1 8ST
Email: mic.wilkinson@northumbria.ac.uk

Thank you for taking the time to read this information sheet. If there are any parts you do not understand please do not hesitate to contact

Contact for further information:

Researcher email: s.j.marshall@northumbria.ac.uk

Supervisor email: kevin2.thomas@northumbria.ac.uk

Name of another person who can provide independent information or advice about the project

Dr Mick Wilkinson, mic.wilkinson@northumbria.ac.uk

Northumbria University NEWCASTLE

Faculty of Health & Life Sciences

INFORMED CONSENT FORM

Project Title: The influence of sex and the oral contraceptive pill on markers of exercise induced muscle damage and the repeated bout effect, following maximum, single limb, knee extension exercise

Principal Investigator: Mr. Steven Marshall

	Please tick or initial where applicable
I have carefully read and understood the Participant Information Sheet.	☐
I have had an opportunity to ask questions and discuss this study and I have received satisfactory answers.	☐
I understand I am free to withdraw from the study at any time, without having to give a reason for withdrawing, and without prejudice.	☐
I give my permission for the still and moving images obtained during the scanning procedures to be used for analysis and reproduced for all scientific literature pertaining to the study.	☐
I agree to take part in this study.	☐

I agree that the following tissue or other bodily material may be taken and used for the study:

Tissue/Bodily Material	Purpose	Removal Method
Blood	For estrogen and progesterone concentration analysis	Venepuncture
Blood	For creatine kinase analysis	Capillary sample or venepuncture

I understand that if the material is required for use in any other way than that explained to me, then my consent to this will be specifically sought. I understand that I will not receive specific feedback from any assessment conducted on my samples, but should any kind of abnormality be discovered then the investigator will contact me.

I understand that the University may store this tissue in a Licensed Tissue Bank only for the duration of the study, it will then be destroyed.

Method of disposal: Clinical waste

Signature of participant..... Date.....

(NAME IN BLOCK LETTERS).....

Signature of researcher..... Date.....

(NAME IN BLOCK LETTERS).....

APPENDIX 2 – SCREENING AND MENSTRUAL CYCLE QUESTIONNAIRE

The influence of sex and the oral contraceptive pill on markers of exercise induced muscle damage and the repeated bout effect, following maximum, single limb, knee extension exercise

The questionnaire will take you approximately 20 minutes to complete. The questionnaire consists of tick boxes or one-sentence answers. If you would prefer not to answer certain questions, please leave them blank. You can take the questionnaire away with you to allow you to answer the questions in private. If you need any help answering the questions, please do not hesitate to contact the Principal Investigator. Please see contact details at the end of the questionnaire.

Weight (Kg):_____**Kg** **Height (m):** _____ **m**
Date of Birth (dd/mm/yy): __/__/__ **Sex:** Male / Female

1. **How would you best describe your ethnicity?**

☐ White ☐ Black or Black British - Caribbean
☐ Black or Black British – African ☐ Asian or Asian British – Indian
☐ Asian or Asian British – Pakistani ☐ Asian or Asian British – Bangladeshi
☐ Chinese ☐ Other Asian Background
☐ Other Ethnic Background

If other, please elaborate _____

_____

2. **Does your job / lifestyle require lifting heavy objects on a regular occasion?**
   For example: Do you lift heavy boxes on and off shelves daily?

   Yes ☐ No ☐

3. **Are you currently competing in University level sport?**

   Yes ☐ No ☐

   If yes, which sport: _____

4. **Are you currently taking part in regular resistance training (weight training)?**

Yes ☐ No ☐

If yes, how often do you complete the following resistance sessions?

Please all that apply *frequency per week*

Upper body ☐ _____

Lower body ☐ _____

Mixed upper and lower ☐ _____

Plyometric (repeated jumps) ☐ _____

5. **Have you <u>ever</u> taken anabolic steroids?**

Yes ☐ No ☐

6. **Have you <u>ever</u> smoked?**

Yes ☐ No ☐

If you answered no, please go to question 8.

7. **How often did you / do you smoke?**

Please tick one *nº cigarettes/day*

Every day ☐ _____

Every other day ☐ _____

Twice a week ☐ _____

Once a week ☐ _____

8. **Do you drink alcohol?**

Yes ☐ No ☐

If yes, how many units a week do you drink?

For example: A small wine (175ml) = 2.3 units, a pint of larger = 2.3 units, a single (25ml) measure of spirits = 1 unit, alcopop (275ml) = 1.1 units

_____ units in a week

9. Do you suffer from any medical conditions?
For example: Arthritis, Myositis, Fibromyalgia, Myopathy, Diabetes Mellitus, or Hypothyroidism

Yes ☐ No ☐

If yes, please elaborate: _____

_____

_____

10. Are you currently taking any medication?

Yes ☐ No ☐

If yes, please elaborate: _____

_____

11. Are you currently taking any supplements or vitamins?
For example: Protein supplements or vitamin e tablets?
Yes ☐ No ☐

If yes, please elaborate: _____

_____

12. Have you <u>ever</u> been diagnosed or suffered from problems with your tendons?
For example: Tennis elbow, Tendinitis, Avulsion (torn tendon).

Yes ☐ No ☐

If yes, please describe information regarding the type of condition / injury, when it occurred (dd/mm/yy) and what tendon/s are affected:

_____

_____

_____

13. Are you <u>currently</u> suffering from any musculoskeletal or tendon injury?
For example: Sore muscles, broken bones or sore tendons

Yes ☐ No ☐

If yes, please describe information regarding the type of injury, injury location, and when it occurred (dd/mm/yy):

_____

_____

_____

14. Have you <u>ever</u> severely controlled your diet to achieve a dramatic change in weight?

Yes ☐ No ☐

FEMALE PARTICIPANTS ONLY

15. Do you have any children?

Yes ☐ No ☐

If no, please go to question 17

a. Please list the date/s you had your child/ children (dd/mm/yy):

1) ___/___/___
2) ___/___/___
3) ___/___/___
4) ___/___/___
5) ___/___/___

16. Have you breast fed in the last year?

Yes ☐ No ☐

17. At what age did you start your period?

_____ years old

18. On average how long does your menstrual cycle last (how many days between a period)?

_____days

19. On average how long does your period last?

_____ days

20. Have you had a regular period for the last six months (one period at least every month and no spotting in-between periods)?

Yes ☐ No ☐

21. Have your periods stopped?

Yes ☐ No ☐

If no, please go to question 22

a. What was the date of your last period (dd/mm/yy)?

___/___/___

22. Have you ever had a hysterectomy?

Yes ☐ No ☐

23. Have you ever had your ovary / ovaries removed?

Yes ☐ No ☐

24. Have you ever taken the oral contraceptive pill?

Yes ☐ No ☐

If no, please go to question 25

a. What type of pill did / do you take (name and dose of oestrogen and progesterone)?
Dose values can be found on the medication box. If you are unsure, please bring your medication with you.

_____

_____

b. When did you start taking the pill (mm/yy)? ____/___
c. If you have, when did you stop taking the pill (mm/yy)? ____/___

25. Have you ever used any other form of hormone-based birth control (injection, vaginal ring, etc.)?

Yes ☐ No ☐

If no, please go to question 26

a. What type of hormone-based birth control did / do you take (name and dose of oestrogen and progesterone)?
Dose values can be found on the medication box. If you are unsure, please bring your medication with you.

_____

_____

b. When did you start using the contraception (mm/yy)? ____/___
c. If you have, when did you stop using the contraception (mm/yy)? ____/___

26. Have your ever used hormone replacement therapy?

Yes ☐ No ☐

Thank you for your time and honesty whilst completing the questionnaire. Be assured all questionnaires will remain strictly confidential.

Contact details:

Steven Marshall (Principal Investigator)
s.j.marshall@northumbria.ac.uk